# GENETIC DIVERSITY AND FUNGICIDE SENSITIVITY OF *RHIZOCTONIA SOLANI* ASSOCIATED WITH SOYBEAN SEEDLING DISEASE

BY

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DISSERTATION

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## ABSTRACT

*Rhizoctonia solani*, a ubiquitous and genetically diverse fungus, is an important seedling pathogen of soybean in North America. With the dearth of commercial soybean varieties marketed for resistance to members of this species, seed treatment fungicides have become increasingly important as a management option. However, our knowledge of the groups associated with soybean seedlings, the genetic structure of the pathogenic groups, as well as their potential for adaptation to fungicide seed treatments, is very limited. To bridge this knowledge gap, we characterized, using conventional and molecular techniques, *Rhizoctonia* isolates collected from farmers' fields in the U.S. and in Canada to identify the predominant and most aggressive groups. Three taxonomic groups were identified: *R. solani*, *R. zeae*, and the binucleate *Rhizoctonia*. The *R. solani* isolates comprised members of anastomosis groups (AG) 2-2IIIB, 3PT, 4 HGI, 4 HGIII, 7, and 11. Isolates of AG-2-2IIIB were the most frequently recovered and the most aggressive on soybean and corn. Using single nucleotide polymorphism markers identified from genotyping-by-sequencing approach, the genetic structure of the populations of AG-2-2IIIB from Illinois, Ohio, and Ontario was assessed for clues about the pathogen's reproductive biology and to determine if the pattern of genetic variation within populations is consistent with that of a pathogen that is at a high risk of adapting to repeated fungicide applications. While the Illinois population was mostly clonal, the genetic structure of the AG-2-2IIIB populations from Ontario and Ohio revealed a mixed reproductive mode, suggesting the need for caution when applying fungicides. Our results also presented genotype flow as a predominant force shaping the population genetic structure of this AG. To determine if *R. solani* populations are becoming less sensitive to the fungicide classes commonly used to manage

seedling disease, a fungicide resistance monitoring program was initiated to compare the sensitivities of historical isolates with no prior fungicide exposure to the sensitivities of isolates that have been exposed to fungicides over time. Results from fungicide sensitivity assays showed that sensitivity to the fungicide classes tested has decreased in comparison to the baseline *R. solani* population, but control of seedling disease caused by *R. solani* was still achieved regardless of in vitro sensitivity.

The appendix chapter of this dissertation presents the results of a separate study evaluating the potential of a three-gene pyramid for improved soybean aphid management . From greenhouse studies evaluating the differential reaction of soybean isolines with different combinations of aphid resistance genes, *Rag1*, *Rag2*, and *Rag3* to four soybean aphid biotypes, the *Rag1/2/3* pyramid was found to be the most effective.

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*To Olusegun Ajayi (“Popsí”) and Olabisi Ajayi (“Mémooo”)*

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# CHAPTER 1: LITERATURE REVIEW


## Summary

*Rhizoctonia solani* Kühn, the most important species within the genus *Rhizoctonia*, is a soil-borne plant pathogen with considerable diversity in cultural morphology, host range, and virulence. Despite its history as a destructive pathogen of economically important crops world-wide, our understanding of its taxonomic relationship with other *Rhizoctonia*-like fungi, incompatibility systems, population biology, host-pathogen interaction, and other molecular aspects of pathogenicity is rather limited. The first section of this review summarizes what is currently known about the taxonomy and systematics, anastomosis groups, mating systems, population biology, and molecular genetics of *R. solani*. The second section provides an overview of the pathology and management of Rhizoctonia root and hypocotyl rot of soybean, a seedling disease of importance in North America.


**Taxonomy:** Domain Eukarya, Kingdom Fungi, Sub-kingdom Dikarya, Phylum Basidiomycota, Subphylum Agaricomycotina, Class Agaricomycetes, Order Ceratobasidiaceae, Family Cantharellales, Genus *Rhizoctonia*.

**Identification:** Traditional identification is based mostly on vegetative characters. Septate multinucleate hyphae appear hyaline when young, but turn brown with age. Hyphal branch originates from distal dolipore septum with a characteristic constriction at the branching point. Monilioid cells and sclerotia of uniform texture are usually produced by most, but not all, members of the species. Conidia, clamp connections, rhizomorphs, and cultural pigmentations other than brown are never observed. Basidiomal structure of sexual state is characterized by a vertically branching hymenium succeeded by layers of elongated basidia slightly wider than basal hyphae.

**Host range and disease symptoms:** *R. solani* attacks members of the Poaceae (e.g. corn, rice, wheat, barley, oat), Fabaceae (e.g. soybean, peanut, dry bean, alfalfa, chick pea, lentil, field pea), Solanaceae (e.g. tobacco, potato), Amaranthaceae (e.g. sugar beet), Brassicaceae (e.g. canola), Rubiaceae (e.g. coffee), Malvaceae (e.g. cotton), *Asteraceae (e.g. lettuce)* and Linaceae (e.g. flax) family. Symptoms on diverse hosts include seed rot, root rot, hypocotyl rot, crown rot, stem rot, limb rot, pod rot, stem canker, black scurf, seedling blight, and pre- and post-emergence damping off. Seedling disease symptoms on soybean range from seed rot and pre-emergence damping off, especially under high inoculum density, to root or hypocotyl rot, depending on which anastomosis group is present at the time of infection.

**Management:** Crop rotation appears ineffective due to long-lived overwintering sclerotia and the soybean-corn rotations throughout the U.S. Corn Belt that can exacerbate inoculum build-up. Commercial soybean cultivars marketed for resistance also are lacking, making fungicide seed treatments the disease control method of choice.

## Introduction

*Rhizoctonia solani* is a ubiquitous soil-borne necrotroph that inflicts damage on a wide range of economically important crops. Considerable diversity in colony morphology, biochemical and molecular markers, pathogenicity, and virulence exist among members of this species, which has permitted their classification into fourteen somatically incompatible groups otherwise termed anastomosis groups (AGs). Although a plethora of biochemical and molecular genetic studies confirm traditional AG classification and recognize AGs as non-interbreeding populations (Anderson 1984) with distinct evolutionary origins (Vilgalys and Cubeta 1994), the genetic basis and control of the observed incompatibility, as well as the factors that shape population structure within each AG remains unclear. While host specificity occurs within

certain AGs, AG-3 for example (Banville et al. 1996), host range diversity is typical among isolates of this species. Isolates of AG-2-2IIIB, AG-4, and AG-5 have been associated with seedling disease of soybean in the north central United States (Liu and Sinclair 1991; Muyolo et al. 1993a; Fenille et al. 2002; Sneh et al. 1991; Bolkan and Ribeiro 1985; Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996; Zhao et al. 2005a). Seedling blight, root rot and hypocotyl rots are typical symptoms observed when susceptible genotypes are planted on high-risk fields and in conditions favorable for disease development (Yang 1999). Unfortunately, our understanding of the mechanisms promoting infection and other aspects of host-pathogen interaction is limited, impeding the development of resistant genotypes. An overview of the taxonomy and genetics of *R. solani* and a brief review of the pathology and management of root and hypocotyl rot of soybean, a seedling disease of importance in the North America, is presented.

### ***Rhizoctonia*: genus description and taxonomic classification**

While studying the pathology of an unknown fungus that attacked alfalfa (*Medicago sativa*) and saffron (*Crocus sativus*), De Candolle (1815) first described the genus *Rhizoctonia*. He reported two species within this genus: *R. crocorum* affecting saffron, and *R. medicaginis* affecting alfalfa. According to De Candolle, the two basic characteristics of the genus are the production of sclerotia other than those produced by *Sclerotium*, and the association of the mycelia with the roots of plants. However, the lack of definitive morphological characteristics that would have aided proper identification of species belonging to the genus led, in earlier years of *Rhizoctonia* taxonomy, to the classification of unrelated fungi in the genus *Rhizoctonia* (Parameter 1970). The *Rhizoctonia* species complex consists of a group of genetically diverse eukaryotic organisms belonging to the kingdom Fungi, sub-kingdom *Dikarya*, phylum

*Basidiomycota*, sub-phylum *Agaricomycotina*, class *Agaricomycetes*, and different orders and families (CABI database (www.indexfungorum.org; www.mycobank.org)). The genus *Rhizoctonia* includes a ubiquitous group of fungi that are naturally occurring in both cultivated (Ogoshi 1987) and uncultivated areas of different parts of the world, causing diseases symptoms such as damping off, root rot, seed decay, fruit decay, stem cankers, and foliage diseases on a wide range of host plants (Menzies 1970). They exist as both plant pathogens, inflicting economic losses on important agricultural crops, and as saprophytes surviving on dead organic matter in the soil. They have also been found to exist as endophytic symbionts on orchids and mosses (Currah et al. 1987; Warcup and Talbot 1966).

In the past, fungi classified within the anamorphic (asexual) genus *Rhizoctonia* consisted of species having different teleomorphic (sexual) genera and belonging in different families and orders, and the most important teleomorphic genera causing plant diseases and of greatest interest to plant pathologists included *Thanatephorus* Donk (Anamorph: *R. solani*), *Ceratobasidium* Rogers (Anamorph: *Ceratorhiza*), and *Waitea* Warcup and Talbot (Anamorph: *R. zeae*) (Vilgalys and Cubeta 1994). *Thanatephorus* and *Ceratobasidium* are members of the family *Ceratobasidiaceae* and order *Cantharellales*, while *Waitea* is classified under the family *Corticiaceae* and order *Corticiales* (CABI database: www.indexfungorum.org; www.mycobank.org). These three genera can be distinguished using morphological features such as the mycelial hyphae and basidial structures. With respect to mycelial features, all three genera lack clamp connections but possess wide monilioid hyphae, sclerotia, and a dolipore septa. The vegetative hyphae of *Thanatephorus* and *Waitea* are consistently wider than those of *Ceratobasidium* (Roberts 1999; Sneh et al. 1991; Stalpers and Andersen 1996), although this may not always hold true as hyphal diameter may vary depending on in vitro conditions

(Andersen 1990). In addition, the young vegetative hyaline hyphae of *Thanatephorus* become brown with age, while the mature hyphae of *Waitea* are salmon in color (Sneh et al. 1991). Brownish sclerotia of *R. solani* are also easily distinguishable from the reddish sclerotia of *R. zeae*. The presence of multinucleate cell compartments in *Thanatephorus* and *Waitea* is also frequently used in differentiating them from the binucleate *Ceratobasidium* (Andersen 1996; Parmeter et al. 1967). The basidiomal structure of *Thanatephorus* is morphologically similar to *Ceratobasidium*; however, the former is characterized by a vertically branching hymenium succeeded by layers of elongated basidia slightly wider than basal hyphae, while the latter has laterally branching hymenium with less elongated basidia that is usually twice the size of the basal hyphae (Roberts 1999; Stalpers and Andersen 1996).

A number of different epithets (species and subspecies levels) of the genus *Rhizoctonia* have been described by different authors (Ogoshi 1996); however, many of these taxa do not belong in the genus. Andersen and Stalpers (1994) observed all type materials and live cultures for sterile mycelium and sclerotia (not similar to that produced by *Sclerotium*), as well as protologues of published epithets. From their work, only seven taxa were considered to conform to *Rhizoctonia* DC *sensu lato*. The others were either synonymous with previously described epithets, invalidly published, or did not conform to the descriptions of *Rhizoctonia*. Of the 109 species of *Rhizoctonia* currently available in fungal databases (www.indexfungorum.org; www.mycobank.org), only 64 are reported to have been validly published, with the others considered invalid or lacking a proper description or reference (nomen nudum).

**Classification of *Rhizoctonia* species**

*Rhizoctonia* spp. generally do not produce conidia; however, sexual spores, which are rarely obtained in culture and infrequently observed on host tissues, are sometimes produced.

This characteristic feature could have possibly contributed to the late correlation of the teleomorph stage with previously identified anamorphs (Warcup and Talbot 1966, 1980). Due to this lapse, initial studies on *Rhizoctonia* focused mainly on the anamorphs utilizing morphological and cytological techniques for identification purposes. Several methods that permitted taxonomic differentiation at both genus and species levels included the use of cellular nuclear number, colony morphology, anastomosis grouping (Parmeter et al. 1969), structure of septal pore (Moore 1987), various biochemical analyses (Cruickshank 1990; Mordue et al. 1989; Sweetingham et al. 1986), and more recently, molecular techniques. Based on cellular nuclear number, *Rhizoctonia* spp. have been divided into the binucleate group (genus *Ceratobasidium*), with two nuclei per cell, and the multinucleate group (genera Thanatephorus and Waitea), having three or more nuclei per cell (Burpee et al. 1980; Parmeter et al. 1969). The correlation of anamorphic states with their respective teleomorphs was necessary at the early stage of *Rhizoctonia* research to unravel the extent of diversity within the genus and to aid rapid identification. Using the ultrastructure of the septal pore to accomplish this, Moore (1987) divided the genus into several anamorphic genera: *Rhizoctonia* (*Helicobasidium*), *Moniliopsis* (*Thanatephorus and Waitea*), *Ceratorhiza* (*Ceratobasidium*), and *Epulorhiza* (*Tulasnella and Sebacina*). *Helicobasidium* is the teleomorph of the type species *R. crocorum* originally described by De Candolle (Moore 1987), and *Moniliopsis* (especially *Thanatephorus*) and Ceratorhiza (*Ceratobasidium*) are by far the most studied genera (Ogoshi 1996). Isolates having the *Helicobasidium* teleomorph were found to have simple septa similar to fungi classified in the Uredinales (Bourett and McLaughlin 1986), while other teleomorphic genera of *Rhizoctonia* have complex dolipore septa (Moore 1987). To differentiate the three remaining genera, Moore (1987) defined three new anamorphic genera: the anamorph genus *Epulorhiza* for those

*Rhizoctonia* species with 'imperforate parenthesomes', *Ceratorhiza* for those having parenthesomes with large perforations and having binucleate *Ceratobasidium* as their teleomorph, and *Moniliopsis* for *Rhizoctonia* species having similar perforated parenthesomes as *Ceratorhiza* but having multinucleate *Thanatephorus* (*R. solani*) and *Waitea* as their teleomorphs. However, the preservation of the anamorph *Rhizoctonia solani* against *Moniliopsis* was proposed (Stalpers et al. 1998) due to its widespread usage among researchers working on *Rhizoctonia*-like fungi.

***Rhizoctonia solani***

*Rhizoctonia solani* (synonym: *Thanatephorus cucumeris* Donk), the most studied species within the genus (Sneh et al. 1991), was erected and described by Kühn (1858) for a fungus he observed on diseased potato tubers. Kühn's description of the species, as well as the disease symptoms it caused on the potato tubers, appeared vague with some elements of contamination, based on his report on “spore-like bodies” (Parmeter and Whitney 1970). This lack of clarity in fungal identification, coupled with a non-existent type material that could have allowed for a proper identification of the fungus, resulted in some confusion among later scientists who described species within the genus *Rhizoctonia* (Duggar 1915). In an attempt to aid proper identification of the species and to clear the confusion among pathologists working on *Rhizoctonia*-like fungi, Duggar (1915) did a thorough review of the species concept, providing detailed information on the important morphological characteristics that distinguished it from other species, as well as a description of the kinds of diseases it causes. Based on the work of Duggar and Parmeter and Whitney (1970), the defining characteristics of *R. solani* include septate hyphae, multinucleate cells in young hyphae, brown coloration of mature hyphae, right angled hyphal branching, constriction at the point of branching, dolipore septa that permits

unrestricted cell to cell movement of cytoplasm, mitochondria and nuclei, production of monilioid cells, and sclerotia of uniform texture (see Fig. 2.1 of chapter 2). Conidia, clamp connections, rhizomoprhs, hyphal pigmentations other than brown, and sexual states other than *T. cucumeris* are characters never observed. A few years after the re-establishment of the basic features of *R. solani*, Ogoshi (Ogoshi 1975, 1987) re-defined the genus concept of *Rhizoctonia* by incorporating a few of the characteristic features defining *R. solani*. As a result, the present-day consensus on the defining characteristic of the genus *Rhizoctonia* include: hyphal branches emanating from the distal septum of young vegetative hyphae; apparent constriction at the point of branching; the presence of a septum at the point of origin of hyphal branches; dolipore septum; absence of conidia, clamped hyphae, and rhizomorphs; and the presence of sclerotia that are not differentiated into rind or medulla.

**Current taxonomic classification of *Rhizoctonia solani***

Over the years, many fungi, including the plant pathogenic groups, have been assigned more than one scientific name due to the identification of the asexual and sexual stages at different times or periods during their discovery. The nomenclatural system for these pleomorphic fungi have, no doubt, become a subject of debate among mycologists as recent molecular data have begun to highlight the weaknesses of using morphological characters to assign scientific names to fungi. A few years after a proposal was made to eliminate the dual system of nomenclature and to adopt a system that reflects phylogeny (Rossman and Samuels 2005), changes were made at the "One fungus = One Name" symposium held at the Amsterdam, the Netherlands in 2011 (Hawksworth 2011). The new rule, which follows the "principle of priority" set forth by the International Code for Botanical Nomenclature (ICBN), and which was to become effective as of January 1, 2013, requires that precedence be given to the first validly published generic name of

a fungus, regardless of whether the documented or named type species is an anamorph or teleomorph. However, in cases where the most widely recognized name for that fungus was later published, it can still be considered the name of choice, provided it is approved by the mandated body.

Going by these new taxonomic rules, the correct current name for the most studied species within the genus *Rhizoctonia* should be straightforward. For the anamorph *R. solani* with teleomorph *Thanatephorus cucumeris*, *Rhizoctonia* was first described by De Candolle (1815), while *Thanatephorus cucumeris* was described much later by Donk (1956). *R. crocorum* was designated the original type species of *Rhizoctonia*, although changes were later made to replace it with *R. solani* (Stalpers et al. 1998) due to the presence of a simple septal pore structure different from the dolipore septa characteristic of a true *Rhizoctonia* (Moore 1987) and partly due to the widespread use of *R. solani*. Interestingly, in the available fungal repository, there appears to be some disagreement on the current name of this fungus. The USDA host database indicates *R. solani* as the current name (https://nt.ars-grin.gov/fungaldatabases/new_allView.cfm?whichone=Nomenclature&thisName=Rhizoctonia%20solani&fromallCount=true&organismtype=Fungus) while the Index Fungorum database indicates *T. cucumeris* as the current name (http://www.speciesfungorum.org/Names/NamesRecord.asp?RecordID=229666) .

**Population biology and genetics of *Rhizoctonia solani***

**Reproduction in *R. solani* and mechanism of variation**

In an attempt to unravel the basis for the variation observed in this species, Flentje et al. (1963, 1970) studied the behavior of the nucleus during mitotic and meiotic division. They provided a detailed description of the processes leading up to the division of multinucleate

vegetative cells and those involved in the formation of sexual spores from vegetative structures. Briefly, in vegetative hyphae, nuclear division is mostly conjugate, implying that simultaneous division of all nuclei occurs within each cell, producing twice the number of daughter cells as the dividing nuclei, half of which are observed to migrate towards the newly developing side branch, with a septa separating the other half as they move towards the main hyphae. Unequal separation of daughter nuclei during mitotic division in individual cell compartments is suggested to likely account for the differences in the number of nuclei observed between young and older cells. Nuclear division appears to occur in synchrony across all cells.

To initiate sexual reproduction, genetically compatible nuclei present in multinucleate vegetative cells pair up at the initial stages resulting in the formation of basidia, the cells on which the sexual spores (basidiospores) are later borne. This pairing produces binucleate prebasidial cells that are separated by septa. Upon formation of basidia, paired nuclei within each basidium undergo karyogamy, a process involving the fusion of sexually compatible nuclei to form diploid nuclei. Eventual meiosis of diploid nuclei results in the formation of four haploid nuclei that later develop into uninucleate basidiospores borne on the basidium by means of four stalk-like structures called sterigmata. As is the convention in other basidiomycetes, plasmogamy of germinated uninucleate spores produces multinucleate cells.

Field isolates of *R. solani* are mostly heterokaryotic (hetero = different; karyon = nucleus), implying the presence of at least two genetically different nuclei within each multinucleate cell compartment. Heterokaryon formation, possibly due to plasmogamy between genetically different homokaryotic (homo = similar; karyon = nucleus) mycelia, has been implicated as a possible mechanism of variation in this species (Flentje and Stretton 1964). Evidence to prove the heterokaryotic nature of *R. solani* first came from experiments conducted

by Whitney and Parmeter (1963) and Anderson et al. (1972), where single-spore cultures of field isolates were induced to form heterokaryons showing cultural characteristics different from those of parent homokaryons. Further support for heterokaryon formation was later presented by Bolkan and Butler (1974), where heterokaryotic field isolates were observed to not only form morphologically and pathogenically distinct heterokaryons when paired with compatible heterokaryons, but were also found to form viable heterokaryons when paired with compatible homokaryons. In addition to heterokaryosis, sexual recombination (meiosis) and mutation also can contribute significantly to genetic variation in *R. solani* (Flentje and Stretton 1964), although evidence for these two mechanisms under natural field conditions is limited. Studies elucidating the role of sexual recombination in creating diversity mostly have been hampered by the inability to induce, under laboratory conditions, sporulation of most field isolates for genetic analysis. This is due partly to the predominant functionality of the species as an asexual organism in nature (Adams 1996; Cubeta and Vilgalys 1997), as well as the self-sterile condition of most field isolates (Ogoshi 1987).

**Anastomosis groups (AG) and heterokaryon formation in *R. solani***

The striking variation in colony morphology, host range, virulence, nutritional requirement, and other characteristics of isolates recognized as *R. solani* has led to a system of classification based on hyphal anastomosis. This classification system first was used to classify isolates of this species about eight decades ago (Matsumoto et al. 1932), and it since has been modified by several authors (Parmeter et al. 1969; Richter and Schneider 1953) to produce the present-day concept of AGs. Anastomosis grouping has not only served as the single most important criterion for delimiting isolates recognized as members of the species, it also has revealed that *R. solani* is a species complex consisting of non-interbreeding (Anderson et al.

1972) or reproductively isolated (Anderson 1984) populations. Using this classification system, genetically similar isolates that readily undergo hyphal fusion and cytoplasmic and nuclei exchange are placed in the same AG, while isolates that fail to achieve hyphal contact, hyphal fusion, and nuclear exchange are considered members of different AGs.

Conventional methods of determining the AG of an isolate involves pairing an unknown isolate with a known tester isolate on 2% water agar (Ogoshi 1975; Parmeter et al. 1969; Rovira et al. 1986); on a cellophane membrane placed over nutrient agar (Parmeter et al. 1969); on bare glass slides incubated in moist chambers (Kronland and Stanghellini 1988); or on agar coated slides placed on moist filter paper in petri dishes (Martin and Lucas 1984). Hyphae of paired isolates are allowed to grow towards each other and the different reaction types observed microscopically are used to determine if the unknown isolate belongs in the same AG as the tester isolate. Different staining techniques for observing hyphal interactions have been described extensively (Kronland and Stanghellini 1988; Kulik and Dery 1995).

Earlier studies on anastomosis reaction identified different reaction type categories that can be observed when isolates are paired. Terminologies such as perfect fusion, imperfect fusion, contact fusion, no reaction, killing reaction, wall fusion, and cell death (Flentje and Stretton 1964; Matsumoto et al. 1932; Parmeter et al. 1969) were used to define and characterize each reaction type category. However, to provide clear-cut characteristics defining each category, Carling et al. (1988) developed a new classification system involving four reaction types, C0, C1, C2, and C3 (see Fig. 2.2 of chapter 2). In the C0 reaction type, interaction or contact between hyphae of paired isolates is non-evident. Isolates exhibiting such reaction type are said to be genetically different and belong to different AGs. For the C1 reaction, cell wall contact between hyphae of paired isolates is occasionally observed; however, contact between cell membranes

does not occur, and the death of anastomosing cells may or may not be observed. This form of reaction is mostly observed among distantly-related isolates belonging to the same or different AGs. For genetically distinct isolates within the same AG, cell wall fusion and the death of anastomosing and adjacent cells, otherwise referred to as the "killing reaction" (Flentje et al. 1967), are usually apparent; a characteristic feature defining the C2 reaction type. The last reaction type, C3, is characterized by a perfect fusion of cell walls and cell membranes of paired isolates and by an absence of dead anastomosing and adjacent cells. This is commonly observed during self-anastomosis as well as during fusion among clones. However, because anastomosis reaction alone cannot be used to define clones, the concept of vegetatively compatible population (VCP) was developed to describe isolates of an AG that exhibit the C3 reaction when paired (MacNish et al. 1997).

Currently, fourteen anastomosis groups (AGs 1-13 and AG-B1) have been identified in *R. solani*, and some of the groups (AGs 1, 2, 3, 4, 6, 8 and 9) have been further divided into sub-groups (Table 1.1) on the basis of characteristics other than hyphal anastomosis reactions, with the exception of AG-2 which is subdivided on the basis of the frequency of fusion between isolates. AG-1 isolates are divided into six subgroups, AG-1-1A (sheath blight), AG-1-1B (web blight), AG-1-1C (damping off), AG-1-1D (necrotic leaf spot on coffee), based on pathogenicity and cultural morphology, and AG-1-1E and AG-1-1F, based on polymorphisms at the internal transcribed spacer regions of the ribosomal RNA (rRNA) genes (Kuninaga et al. 2002). AG-2 has been designated the most heterogeneous of all fourteen AGs. Initial subdivision identified two subgroups, AG-2-1 (crucifer type) and AG-2-2 (canker pathogens of sugar better and carrot), on the basis of frequency of hyphal fusion, with greater affinity for hyphal anastomosis observed within, than between, subgroup; however, hyphal anastomosis reactions might not be a

dependable method for differentiating subgroups of AG-2 (Carling et al. 2002). AG-2-1 isolates are autotrophic for thiamine, while AG-2-2 isolates are thiamine auxotrophic (Ogoshi 1987). Three subgroups exist within AG-2-1: AG-2-1, AG-2t (tulip isolate), and AG-2Nt (tobacco isolate). Based on pathogenicity, cultural morphology, isozyme analysis (Liu and Sinclair 1992), cellular fatty acid composition (Stevens-Johnk et al. 1993), and sequence analysis of the ribosomal DNA ITS region (rDNA-ITS), five subsets can be differentiated within AG-2-2: AG-2-2IIIB, AG-2-2IV, AG-2-2LP, AG-2-3, and AG-2-4. Temperature requirements can be used to differentiate AG-2-2IIIB ('mat rush' type) and AG-2-2IV isolates ('sugar beet' type); the former would grow at 35°C, while the latter would not (Sneh et al. 1991). A proposal has been made to classify the "bridging group", AG-BI, as a new subgroup of AG-2 due to its frequency of anastomosis reaction with all subsets of the group (Carling et al. 2002). For AG-3 (solanaceous type), three subgroups have been reported: AG-3 PT (potato type), AG-3 TB (tobacco type), and AG-3TM (tomato type); the first two cannot be differentiated by hyphal anastomosis but by cellular component analysis (Stevens-Johnk et al. 1993) and rDNA-ITS sequence analysis (Kuninaga et al. 2000). Three homogeneous groups (HG) have been identified in AG-4. HG-I and HG-II isolates were initially described and found to differ by DNA base sequence homology (Kuninaga and Yokosawa 1984a) and by sclerotia color on potato dextrose agar (PDA) (Sneh et al. 1991). Vilgalys (1988), using DNA/DNA hybridization, provided further evidence to support heterogeneity within this AG, and the third group, HG-III, was later identified and differentiated from the first two groups based on the analysis of fatty acid (Steven-Johnk and Jones 2001). Within AG-6, homogenous groups (HG-I) and isolates with remarkable genetic variation have been confirmed by morphological characters in culture and by DNA base sequence homology (Kuninaga and Yokosawa 1984b). At least five pectic zymogram groups (ZGs), ZG1-1, ZG1-2,

ZG1-4, ZG1-4, and ZG1-5, have been reported in the AG-8 (MacNish and Sweetingham 1993); while two subgroups, thiamine prototrophic (TP) and thiamine auxotrophic (TX), are evident in AG-9 (Carling and Kuninaga 1990). Subgroups within AG-5, AG-7, AG-10, AG-11, AG-12, AG-13, and AG-BI have not been reported. Interestingly, members of different AGs of *R. solani* have, in the past, been found to have *Thanatephorus cucumeris* as their teleomorphs (Carling et al. 1994; Carling et al. 1987; Homma et al. 1983; Hyakumachi and Ui 1987; Kuninaga et al. 1978; Neate and Warcup 1985; Ogoshi 1975, 1984), and differentiation based on sexual characters has not been reported. According to Ogoshi (1987), this inability to differentiate AGs by sexual state may be one of the many reasons *R. solani* is still regarded as a single species.

The concept of anastomosis grouping also has been extended to other species of *Rhizoctonia*, including the binucleates with teleomorph *Ceratobasidium* and the multinucleate with teleomorph *Waitea*. Species within the genus *Ceratobasidium* have been divided into AGs by scientists in the United States and Japan. The former reported seven *Ceratobasidium* anastomosis groups (CAGs): CAG-1 to CAG-7 (Burpee et al. 1980), and the latter reported 17 AGs: AG-A to AG-Q (Ogoshi et al. 1983a, b). However, as five of Japan's CAGs were indistinguishable from a few of the United States' AGs, and the remaining two CAGs appeared not to correspond with any of the United States' AGs, the Japanese system of classification has been adopted and modified to comprise 21 AGs for the binucleate *Rhizoctonia* (Sneh, Burpee, and Ogoshi 1991). For *Waitea*, two *Waitea* anastomosis groups (WAGs), WAG-O (*W. circinata* var. *oryzae;* anamorph *R. oryzae*) and WAG-Z (*W. circinata* var *zeae*; anamorph *R. zeae*), have been reported (Oniki et al. 1985).

**Incompatibility systems and population structure in *R. solani***

Although the genetic basis of anastomosis in *R. solani* is not fully understood, it has been described as a somatic or heterogenic incompatibility system (Anderson 1984; Worrall 1997) that limits the fusion of genetically different individuals/isolates of a species, thereby preserving the identity of each individual (Rayner 1991) and ensuring a stable heterokaryon (Anderson 1982). Genetic studies aimed at understanding the mechanism behind heterogenic incompatibility in *R. solani* are lacking (Anderson 1984), but studies from other basidiomycetes indicate control by many genes with multiple alleles (Anderson 1984; Rayner 1991), with genetic differences at governing loci resulting in incompatibility (Worrall 1997). In addition, genetic loci controlling somatic incompatibility in basidiomycetes have, in many cases, been shown to be unlinked with those associated with mating compatibility (Hansen et al. 1993; Rizzo et al. 1995). In heterokaryotic field isolates of *R. solani*, the definitive "killing" or C2 reaction type observed microscopically is indicative of heterogenic or somatic incompatibility, a genetic system which functions to prevent cytoplasmic and nuclear exchange between interacting hyphae. Macroscopically, this has been viewed as a "barrage reaction" (Esser 2006) or a line of demarcation between paired isolates on a suitable medium and is often referred to as mycelial incompatibility (Worrall 1997). Heterogenic incompatibility is not only restricted to heterokaryotic field isolates of *R. solani*, but also may occur in heterokaryon-homokaryon and homokaryon-homokaryon pairings (Anderson 1984). The absence of a "killing reaction" observed during self-anastomosis provides strong evidence for genetic distinctiveness of isolates that produce a C2 reaction when paired; however, one cannot conclude in the absence of diagnostic and reliable markers that isolates producing a C3 reaction are of one and the same genotype. In ascomycetous fungi, heterogenic incompatibility is operative in both sexual and

asexual phases; however, in basidiomycetes, which are generally known to lack differentiated sex organs, heterogenic incompatibility is only operational at the vegetative phase, thereby precluding sexual reproduction (Esser 2006). In *R. solani*, heterogenic and homogenic incompatibility are known to act independently of each other (Julian et al. 1996). As heterogenic incompatibility functions to prevent fusion of genetically different secondary (heterokaryotic) mycelia, homogenic incompatibility, which controls sexual mating, functions to limit the formation of a heterokaryon from two primary (homokaryotic) mycelia carrying similar alleles at the mating type locus (Worrall 1997). While heterogenic incompatibility restricts outbreeding by preventing the occurrence of genetically different nuclei within a common cytoplasm, homogenic incompatibility promotes outbreeding and subsequent recombination events (Esser 2006) necessary for diversity.

Homogenic or heterokaryon incompatibility systems in *R. solani* have been studied extensively (Anderson et al. 1972; Puhalla and Carter 1976; Whitney and Parmeter 1963; Yang et al. 1992) , and a bipolar mating system – in which a single locus with multiple alleles controls sexual compatibility – is reported to be evident in AGs 1, 4, and 8 (Anderson et al. 1972; Yang et al. 1992 ). In AG-4, two closely linked genes, collectively termed heterokaryon incompatibility factor (H factor), were identified as genetic determinants for homogenic incompatibility, and 17 different H factors are estimated to exist in natural populations (Anderson et al. 1972). Successful heterokaryon formation occurs between paired homokaryons carrying different H factors, and heterokaryotization is characterized by the production tufts of heterokaryotic hyphae at the junction of pairing, although subsequent studies indicated the non-reliability of tuft production as a measure of heterokaryon formation (Cubeta et al. 1993). The contribution of H factors to outbreeding, nuclear pairing, and heterokaryon stability also has been recognized

(Anderson et al. 1972; Puhalla and Carter 1976). Interestingly, heterokaryosis was observed to be restricted to compatible homokaryons within the same AG, as homokaryons of AG-1 and AG-4, each carrying different H factors, failed to undergo heterokaryosis (Anderson et al. 1972).

Studies on heterokaryosis in *R. solani* can significantly advance our understanding about their mating systems, a concept that has serious implications for population structure (Cubeta and Vilgalys 1997). However, studies expounding mating systems in this species have been hampered by the inability to induce fruiting of field isolates in vitro (Ogoshi 1987), the multinucleate conditions of both homokaryotic and heterokarytic strains, as well as the lack of clamp connections (Cubeta and Vilgalys 1997) – a characteristic feature in all basidiomycetous fungi that have increased our understanding of mating systems. Despite these limitations, a few studies have been successful at deciphering the mating behavior in this species complex. Initial studies reported AG-1 and AG-4 to be homothallic or self-fertile (Anderson et al. 1972), possibly due to homokaryotic fruiting (Adams and Butler 1982). Subsequent experiments validated a predominantly heterothallic bipolar mating system in these AGs (Adams and Butler 1982), and the H factors are now considered to be the mating type locus synonymous with the A and B mating type loci characteristic of basidiomycetes (Adams 1996). Although homothallism has been suggested in AG-2 (Stretton et al. 1967; Cubeta and Vilgalys 1997), both heterothallism and homothallism have been observed in AG-2-2IV (Toda and Hyakumachi 2006). While heterothallism is suggested in AG-8 (Cubeta and Vilgalys 1997), mating systems in other AGs are currently unknown.

Cubeta and Vilgalys (1997) highlighted the paucity of research studies focused on the population genetics of *R. solani*. Genetic structure has been defined as "the amount and distribution of genetic variation within and among populations of a species" (McDonald and

McDermott 1993), and the assessment of the genetic structure of fungal populations has serious implications for predicting how pathogens will evolve in response to management practices. Studies elucidating the population genetic structure of *R. solani* isolates are not only critical for gaining a better understanding of the role of mating and other incompatibility systems in creating diversity, but they also can provide useful information on the risk of development of races, adaptation to resistance genes, and selection of fungicide resistant mutants, a phenomenon that is becoming widespread in other fungal species with increased fungicide use. In *R. solani,* extensive population genetic diversity studies were first accomplished in AG-8 (MacNish et al. 1993; Matthew et al. 1995; Yang et al. 1995). Using isozyme and randomly amplified polymorphic DNA (RAPD) markers, population structure was reported to be predominantly clonal in this AG, although a considerable amount of genotypic variation could exist among isolates recovered from the same geographical location. Evidence for both clonality and sexual reproduction in populations of AG-3 comes from genetic diversity studies utilizing somatic compatibility tests, AG-specific DNA probes (Balali et al. 1996), molecular markers such as simple sequence repeats (SSRs) (Ferrucho et al. 2013), RAPD (Justesen et al. 2003), amplified fragment length polymorphisms (AFLP) (Ceresini et al. 2002a), polymerase chain reaction – restriction fragment length polymorphisms (RFLP-PCR) (Ceresini et al. 2002b), and from the analysis of ITS regions of the ribosomal RNA (Fiers et al. 2011). In AG-1, population structure has been assessed at the subgroup levels, especially in populations AG-1-IA (sheath blight pathogen) affecting rice, maize or soybean in the Unites States, China, Brazil, and other Latin American countries. Initial studies utilizing RFLP markers suggested outcrossing and most likely a heterothallic mating system in populations of AG-1-IA recovered from rice in Texas (Rosewich et al. 1999). Subsequent studies employed microsatellite markers to assess population

structure of both Texas isolates examined by Rosewich et al (1999) and rice- and soybean-infecting isolates recovered in Louisiana (Bernardes-de-Assis et al. 2008). Outbreeding in the Texas isolates was confirmed, and rice-infecting isolates from Louisiana were found to be genetically differentiated from their soybean-infecting counterparts. Population structure of rice-infecting isolates reflected both outbreeding and inbreeding, but mostly inbreeding for soybean-infecting isolates. Interestingly, AG-1-IA isolates from rice in China and Latin America also show mixed reproductive modes (Gonzalez-Vera et al. 2010), while those infecting soybean in Brazil appear to reproduce by a predominantly asexual means (Ciampi et al. 2008). Population structure in AG-4 is primarily outcrossing (Cubeta and Vilgalys 1997), although both sexual and asexual reproduction have been reported in field isolates from Iran (Haratian et al. 2013). While limited information is available regarding population structure in subgroups of AG-2, a mixed reproductive mode has been suggested (Cubeta and Vilgalys 1997).

**Molecular genetics and systematics of *Rhizoctonia solani***

In spite of the tremendous usefulness of traditional anastomosis reaction in identifying *Rhizoctonia* spp. and unraveling the complexity within *R. solani*, its limitations lie in the inability of certain isolates to fuse with tester isolates of any known AG as well as with themselves (Carling 1996; Hyakumachi and Ui 1987; Parmeter et al. 1969). Failure of anastomosis in certain isolates could be attributed to environment and nutrient conditions, mutation, or aging (Hyakumachi and Ui 1987). In such instances, genetic relatedness may not be best defined using anastomosis grouping (Vilgalys and Cubeta 1994). The existence of 'bridging isolates' is another limitation to the use of anastomosis for the correct placement of *R. solani* isolates in homogenous groups. Bridging isolates are able to fuse with isolates from more than one AG (Carling 1996; Sneh et al. 1991). Notably, members of AGs B1, 2, 3, 6, 8, and 11 will

fuse with members of several other AGs, while AGs 1, 4, 5, 7, 9 and 10 will not fuse with isolates belonging to other groups (Carling 1996; Kuninaga et al. 1979). The presence of both non-self-anastomosing and bridging isolates imposes a limitation to the proper identification of certain isolates of this species. These limitations, together with the effort and time it takes to classify isolates microscopically, have been overcome through the development and use of molecular techniques to classify *Rhizoctonia* at the species and AG levels.

Much of the current knowledge relating to our understanding of the diversity within the genus *Rhizoctonia* has come from the use of molecular techniques. Various tools of molecular biology that have been employed for classifying *Rhizoctonia* have all together validated previous classification of *Rhizoctonia* spp. into anastomosis groups and subgroups (Kuninaga 1996; Sharon et al. 2006; Vilgalys and Cubeta 1994). Initial studies employed isozyme analysis (Damaj et al. 1993; Liu and Sinclair 1992; Liu et al. 1990), DNA base sequence complementarity (Balali et al. 1996; Kuninaga 1996; Vilgalys 1988), RAPD (Duncan et al. 1993; Fenille et al. 2002; Toda et al. 1998; Tommerup et al. 1995; Yang et al. 1995), AFLPs (Ceresini et al. 2002; Julián et al. 1999), RFLPs (Kuninaga 1996; Vilgalys and Gonzalez 1990), and RFLP-PCR (Guillemaut et al. 2003). Lübeck (2004) and Sharon et al. (2006) provide an excellent review on the various molecular methods used to characterize species of *Rhizoctonia*. More recently, sequence analysis of the nuclear ribosomal rRNA genes – present in multiple copies of tandem repeats within the genome of all fungi – have received much attention for phylogenic studies of AGs and subgroups (Boysen et al. 1996; Carling et al. 2002; Gonzalez et al. 2001; Kuninaga et al. 1997; Salazar et al. 2000; Salazar et al. 1999) in *R. solani*, and has proven to be a more reliable approach for the rapid identification and classification of unknown isolates (Sharon et al. 2006). The rapidly evolving internal spacer transcribed regions (ITS 1 and ITS 2) allow for between- and within-

species studies of phylogenetic relationships between fungal organisms, and the conserved coding regions that flank the ITS regions permit the development of universal primers for comparative studies, a feat that was accomplished more than two decades ago (White et al. 1990). As a consequence, results from sequencing experiments can be deposited in public databases like the National Center for Biotechnology Information (NCBI) GenBank and European Molecular Biology Laboratory (EMBL), allowing researchers from different laboratories to compare and validate results.

Kuninaga et al. (1997), using the rRNA genes to study diversity in *R. solani*, found a high degree of sequence variability in the ITS regions (although greater in ITS 1 than in ITS 2) compared to the spacer 5.8S region that appears to be highly conserved across all AGs. Sequence variability within subgroups was also found to be lower than that within or between AGs, and AG and subgroup classification of known isolates corresponded well with previous classifications using other molecular markers. Similar results were obtained by Gonzalez et al. (2001); however, monophyly of certain AGs was dismissed based on a combined analysis of ITS and the large sub-unit of the rRNA genes, suggesting that subgroups may represent the basic evolutionary unit in *R. solani*. Although sequence analysis of rDNA has improved our understanding of genetic diversity in *R. solani*, it is not without its attendant limitations. First, an uncertainty regarding fungal identity in repositories is an issue. As deposited sequence data for a specific organism are not subject to validation or verification by other researchers, depositing an incorrectly-named organism is almost inevitable. In other instances, incomplete information about an organism in such databases may hinder correct taxonomic identification of an unknown isolate when conducting a blast search. Second, high mutation rates, mostly insertions and deletions, may render the ITS regions unsuitable for the study of evolutionary relationships in *R.*

*solani* (Kuninaga et al. 1997). Although present in multiple copies, the rDNA repeat units are assumed, due to concerted evolution, to evolve as a single copy, and homogenization is expected to reduce intraspecific variation (Arnheim et al. 1980; Hillis and Dixon 1991). However, lack of homogeneity in the rDNA array in an individual or within a species potentially could invalidate phylogenetic inferences made from such sequences. Interestingly, differences of opinion exist regarding the homogenization of rDNA repeats in fungi (Ganley and Kobayashi 2007; Simon and Weiss 2008), and both intraspecific and intragenomic variation in rDNA sequence has been reported in many fungal and fungal-like organisms including *Fusarium* (O'Donnell and Cigelnik 1997; O'Donnell 1992), *Pythium* (Belbahri et al. 2008) *Laetiporus* (Lindner and Banik 2011), *Saccharomyces* (James et al. 2009), arbuscular mycorrhiza (Pringle et al. 2000), and ectomycorrhizal fungi (Smith et al. 2007). Similarly, heterogeneity in the ITS regions has been reported in AG-1 (Grosch et al. 2007), AG-2 (Pannecoucque and Höfte 2009), AG-3 (Justesen et al. 2003; Kuninaga et al. 2000), and AG-4 (Boysen et al. 1996) of *R. solani*. Considering that concerted evolution is presumed to be maintained by meiotic recombination events, such as unequal crossing over or gene conversion (Li and Graur 1991), considerable heterogeneity in rDNA array in a functionally asexual species like *R. solani* is not unexpected. However, given the multinucleate and heterokaryotic nature of field isolates, as well as the ability for sexual reproduction in certain AGs, elucidating the origin of and factors that promote ITS variation would shed more light on the role of concerted evolution in this species.

## *Rhizoctonia solani* associated with hypocotyl and root rot of soybean

### Survival, infection, disease spread, and pathogenicity and virulence factors

Being a ubiquitous fungus, there is hardly any crop of economic importance on which *R. solani* is not pathogenic or from which it has not been isolated. Twelve of the fourteen AGs

identified to date have been associated with at least one crop of economic importance, and six have been associated with soybean seedling diseases (Table 1.1). *R. solani* causes both aerial and below-ground diseases of soybean (*Glycine max* (L.) Merr.), and the plant parts affected appear to be region-specific. For example, in the rice-growing regions of the southern United States, aerial and web blights caused by AG-1 isolates are important diseases of soybean (Atkins and Lewis 1954; Jones and Belmar 1989; O'Neill et al. 1977; Yang et al. 1990), but in the north central United States, hypocotyl and root rot is a well-established soybean seedling disease (Doupnik Jr 1993; Liu and Sinclair 1991; Muyolo et al. 1993; Nelson et al. 1996; Rizvi and Yang 1996; Tachibana 1968). Farmers in the north central United States plant soybean early in the season to achieve higher yields. However, when planting early, a risk of cooler soils is present. Cool soil conditions will retard seedling growth or delay emergence and increase the vulnerability of emerging plants to infection by soilborne pathogens. Seedling disease symptoms of *R. solani* observed on soybean in such environmental conditions include seed decay, pre- and post-emerging damping off, and hypocotyl rot with a characteristic sunken reddish brown lesion (Fig. 1.1). Seed decay and pre-emergence damping off more often are encountered in fields with high inoculum amounts, or when conditions that negatively affect seedling germination and emergence prevail, such as cool and wet weather. Unlike most other seedling pathogens of soybean, *R. solani* is able to cause infections over a wide range of soil temperature and moisture conditions (Dorrance et al. 2003). Seed decay and pre-emergence damping off generally result in missing stands, and if severe, replanting such fields may be required. Hypocotyl and root rot are common after plants have emerged, and affected plants may or may not be killed. Reddish-brown discolorations on the cortical layer of the lateral roots or on the stem close to the soil line usually are evident on symptomatic plants. Plants with affected roots may exhibit poor lateral

root growth, leading to less vigorous plants with reduced water and nutrient uptake abilities. Such plants appear chlorotic and stunted, and as access to soil water continues to decrease, plants eventually may wilt and die (Hwang et al. 1996). Younger plants are more susceptible to infections since resistance generally increases with age. Nevertheless, unfavorable environmental conditions can predispose older plants to infection and eventual death (Sinclair and Backman 1989). Aside from moisture and temperature conditions, applications of some herbicides have been shown to increase disease severity (Bradley et al. 2002) either through their inhibitive activity on antagonistic micro-organisms, or by reducing plant vigor (Bowman and Sinclair 1989). Planting into fields with a history of the disease is also considered a risk factor. Infections usually are not uniformly distributed since most inocula are localized to certain areas of the field. As a result, yield losses usually are not significant since soybean plants are considered good compensators for reduced stands. However, "mid-season" infections can lower yields significantly due to a decline in the compensatory potential of soybean with age (Hwang et al. 1996). Similarly, widespread inoculum distribution across any given field may cause severe disease outbreaks, reducing plant stands and lowering yields. The impact of seedling diseases caused by *R. solani* on soybean yields in the United States is noteworthy (Koenning and Wrather 2010), and yield reductions up to 48% have been reported (Tachibana et al. 1971).

*R. solani* isolates generally do not produce vegetative or asexual spores, and the role of basidiospores as an inoculum source for the seedling diseases they incite on soybean is unknown. The pathogen is a facultative parasite that is very successful at competing with other soilborne saprophytes. Its survival in the soil is aided by the formation of long-lived "nutrient-independent propagules" called sclerotia (Hoitink et al. 1991). Sclerotia, which arise from undifferentiated hyphae or monilioid cells (see Fig. 2.1 of chapter 2) (Sumner 1996), germinate to form mycelia

and serve as an inoculum source for infection (Keijer 1996). Mycelia also have been shown to play a role in disease spread (Papavizas 1970). Depending on the level of inoculum and virulence of isolates present, different disease types may be encountered in the field (Fig. 1.1). When inoculum levels of virulent isolates are high, seed germination is affected, and those seed that manage to germinate die off almost immediately. Under lower inoculum pressure, roots or hypocotyls of germinated plants become rotted.

For most *Rhizoctonia* infections to occur, sclerotia must first germinate to form mycelia that grow towards the host plant. Mycelial growth usually occurs in response to exudates from host plants, and it is succeeded by hyphal attachment to the host's tissues, hyphal growth along the host's epidermal cell walls, formation of T-shaped branches with appressoria-like infection structures, penetration of host tissues by infection pegs formed from swollen hyphal tips, colonization of host tissue, and eventual seedling collapse (Keijer 1996). *R. solani* is considered to be a necrotrophic pathogen that kills its host in advance of colonization, and the killing activity of most necrotrophic pathogens has been associated with the production of extracellular enzymes or toxins (van Kan 2006). Boosalis (1950), who studied the pathogenicity of *R. solani* on soybean, found that discoloration of host tissue preceded hyphal contact, suggesting that necrotic symptoms are a result of the effect of certain toxic substances secreted by invading hyphae. Wyllie (1962) obtained similar findings and observed that root exudates from soybean plants stimulated mycelial growth of the invading fungal isolate. The ability to produce a number of non-host-specific toxins has been implicated as a possible factor behind the wide host range typical of most necrotrophs, although host-specific toxins have been observed with certain necrotrophs like *Cochlioblous victoriae* (Meehan and Murphy 1947), *Helminthosporium maydi*s (Kono and Daly 1979), *Phyllosticta maydis* (Danko et al. 1984), and *Alternaria alternata* f. sp.

*lycopersici* (Gilchrist and Grogan 1976). The disease-causing ability of *R. solani* has been linked to its production of pectinolytic and cellulolytic enzymes (Bateman 1970). Pectinolytic enzymes are members of a group of cell wall degrading enzymes (CWDEs) known to hydrolyze the pectin component of plants, and they have been associated with pathogenicity of many plant-infecting microbes (Collmer and Keen 1986; Lang and Dörnenburg 2000). Specifically, the implication of polygalacturonases and pectin lyases in the pathogenicity of *R. solani* on several hosts, including bean, cotton and sugar beet (Barker and Walker 1962; Bateman 1963; Brookhouser and Weinhold 1979; Bugbee 1990; Sherwood 1966) has been well established. The role of non-enzymatic toxic metabolites, especially phenylacetic acid (PAA) and their meta- or hydroxyl derivatives, for the pathogenesis of soybean (Mandava et al. 1980), rice (Chen 1958), tomato (Bartz et al. 2012; Chen 1958) and bean (Iacobellis and DeVay 1987) have also been extensively investigated. However, the role of specific CWDE and toxic metabolites in various host-pathogen interactions involving different AGs of *R. solani* is not well understood. Given the host specificity and differences in virulence of most pathogenic AGs, further studies are needed to confidently implicate PAA and CWDE as the sole pathogenicity factors necessary for host infection by *R. solani*.

Studies on the molecular mechanism of pathogenicity and/or virulence of different pathosystems are invaluable for the development of targeted and durable disease control measures. Putative genes associated with pathogenesis in a few *Rhizoctonia*/host pathosystems have been identified. In a comparative gene expression pattern analysis, Rioux et al. (2011) found six putative pathogenesis-related genes showing similar expression patterns in the AG-1/rice and AG-3/potato pathosystems during the early stages of infection, suggesting that pathogenicity genes may be conserved across pathogenic AGs. From their study, a hypothetical

model describing key processes involved in *R. solani* pathogenesis was presented, and putative genes likely to be important for each phase of the model were suggested (Table 1.2). Lakshman et al. (2012) identified, by the analysis of expressed sequence tags (ESTs) developed from mycelia grown under virulent and hypovirulent conditions, putative pathogenicity and virulent factors in an AG-4 isolate, Rs23A. Putative genes associated with pathogenesis included those involved in appressorium formation, plant cell wall degradation, toxin secretion, protection from plant defense, and pathogen invasiveness (Lakshman et al. 2012), similar to putative genes identified by Rioux et al. (2011). From a draft genome sequence of the rice sheath blight pathogen (AG-1A), Zheng et al. (2013) identified a suite of carbohydrate-active enzymes (CAZymes) known to be involved in the assembly and degradation of complex carbohydrates, including pectinases, xylanases and laccases, as well as transporters and several cytochrome P450s. From the same study, three secreted effectors, AG1IA_09161 (glycosyltransferase GT family 2 domain), AG1IA_05310 (cytochrome C oxidase assembly protein CtaG/cox11 domain), and AG1IA_07795 (peptidase inhibitor I9 domain) were identified, representing the first set of effectors ever to be reported in *R. solani*. In AG-8, genes belonging to the CAZymes family, also have been identified (Hane et al. 2014). Unfortunately, studies elucidating the molecular mechanisms of pathogenesis in the *R. solani*/soybean pathosystems are lacking, and this probably has hindered research efforts tailored towards the development of resistant soybean germplasm marketed for commercial use.

## Management of *Rhizoctonia* seedling diseases of soybean

### Anastomosis groups of *R. solani* causing seedling disease of soybean

Different AGs have been reported to be associated with soybean seedling disease. Specifically, isolates of AG 2-2IIIB (Fenille et al. 2002; Liu and Sinclair 1991; Muyolo et al.

1993a; Sneh et al. 1991), AG-4 (Bolkan and Ribeiro 1985; Fenille et al. 2002; Muyolo et al. 1993; Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996; Zhao et al. 2005), and AG-5 (Nelson et al. 1996) are highly pathogenic on soybeans, while those of AG-3 (Nelson et al. 1996), AG-7 (Baird et al. 1996) and AG-11(Carling et al. 1994) have been reported to cause very little damage. While isolates of AG-2-2IIIB are reported to be more aggressive on soybean than AG-4 isolates (Fenille et al. 2002; Zhao et al. 2005a; Muyolo et al. 1993a), Muyolo et al. (1993) observed that AG-2-2IIIB isolates were most aggressive on soybean roots and AG-4 isolates were most aggressive on soybean hypocotyls.

**Host Resistance**

Effective management of seedling pathogens involves adoption of several control measures such as the use of clean certified seeds, rotation to a non-host crop, the use of fungicide seed treatments, and when available, the use of resistant cultivars. For a necrotrophic pathogen like *R. solani* with a wide host range, an integrated disease management approach is no doubt more effective for several reasons. First, the ability to overwinter as long-lived sclerotia in the soil or as mycelia in crop debris is a characteristic feature of the fungus. Second, isolates of an AG can cause severe infections on a wide range of crops, and this has been observed mostly with crops that are maintained in rotation. For example, certain AGs that were pathogenic on soybean caused significant infections on sugar beet, dry bean, mustard, and flax, all of which are grown in rotation with soybean in the Red River Valley of Minnesota and North Dakota (Nelson et al. 1996). In the same study by Nelson et al. (1996), isolates of AG-2-2IIIB were found to produce severe symptoms on corn, an important rotational crop in the soybean growing regions of the north central United States. These characteristics render crop rotation an ineffective management strategy and highlight the need for other effective management options.

In the United States, commercial soybean cultivars purchased by growers are those developed and sold by seed companies and marketed as being resistant to certain pests and diseases (Chawla et al. 2013). Soybean cultivars marketed as resistant to Rhizoctonia root and hypocotyl rot are currently unavailable (Bradley et al. 2001), although germplasm accessions and commercial cultivars with moderate resistance that could serve as potential sources of resistance genes have been identified in greenhouse and field screening assays (Bradley et al. 2001; Muyolo et al. 1993b; Zhao et al. 2005b). Despite the identification of germplasm with partial resistance, putative resistance genes and their respective chromosomal locations have not been identified, making the wide deployment of these potential resistance sources difficult. According to Panella and Ruppel (1996), the genetic control of host plant resistance is one of many factors that greatly influences the decision to develop resistant varieties of cultivated crops. In general, resistance to plant diseases can either be complete, in which resistance is conditioned by single genes otherwise referred to as R-genes, or incomplete, in which resistance is conditioned by multiple genes otherwise referred to as minor genes, quantitative resistance loci (QRL) (Poland et al. 2009), or quantitative trait loci (QTL). R-gene mediated resistance to any Rhizoctonia diseases is lacking (Sweetingham 1996), and resistance to Rhizoctonia root and hypocotyl rot of soybean is quantitatively inherited (Bradley et al. 2005; Zhao et al. 2005b). Several studies have identified QRLs associated with resistance to important seedling diseases of soybean, including those caused by *Phytophthora sojae* (Wang et al. 2010; Weng et al. 2007), *Fusarium virguliforme* (Chang et al. 1996; Iqbal et al. 2001; Njiti et al. 2002), and *Fusarium graminearum* (Ellis et al. 2012). Simple sequence repeat (SSR) markers associated with moderate resistance to Rhizoctonia root and hypocotyl rot have been identified in soybean Plant Introduction (PI) 442031 (Zhao et al. 2005b). However, the three SSR markers identified (Satt281, Satt177, and

Sat245) were associated with resistance to an AG-4 isolate. In general, markers that are developed for one pathotype may not be applicable in locations where other pathotypes occur, except when resistance is conferred by the same gene (Mohan et al. 1997). Although in Illinois, Ohio, and the Canadian province of Ontario, isolates of AG 2-2IIIB have been reported (Dorrance et al. 2003; Liu and Sinclair 1991; Muyolo et al. 1993a; Zhao et al. 2005a), pathogenic isolates belonging in AG-4 have been recovered more frequently from diseased soybean plants in Florida, Iowa, North Dakota and Minnesota (Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996b; Zhao et al. 2005a), as well as Brazil (Bolkan and Ribeiro 1985; Fenille et al. 2002) and Zaire (Muyolo et al. 1993a). AG-5 isolates also have been associated with soybean in Canadian province of Ontario (Zhao et al. 2005a) and Minnesota and North Dakota, although these were found to be less aggressive on soybean than other AGs (Nelson et al. 1996). Given the potential of diverse AG types to cause severe damage on soybean seedlings and the differences in virulence across and within AGs (Bolkan and Ribeiro 1985; Dorrance et al. 2003; Muyolo et al. 1993a; Nelson et al. 1996; Zhao et al. 2005a), it is not unlikely that resistance to these different AGs may be conferred by different genes. In addition, there may be additional genes present in other soybean genotypes beside PI 442031 where the three SSR markers were identified. Another detail that bears mentioning is that in contrast to R-gene mediated resistance, which often is considered to be race-specific, quantitative disease resistance is assumed to be non-race specific (Vanderplank 1968). However, isolate specificity of QTLs has been identified in several pathosystems (Arru et al. 2003; Calenge et al. 2004; Caranta et al. 1997; Darvishzadeh et al. 2007; Marcel et al. 2008), making the possibility of detecting *R. solani* AG-specific QTLs in soybean possible.

## Chemical Control

The dearth of commercial soybean cultivars with high levels of resistance and the limited or non-existent breeding efforts directed towards developing resistant genotypes have precluded the use of resistant soybean cultivars in managing Rhizoctonia root and hypocotyl rot of soybean, making the use of fungicide seed treatments that offer both seed and seedling protection during the initial stages of growth (Dorrance et al. 2003; Kataria and Gisi 1996) a more common method of disease management. Seed treatments not only protect germinating seedlings from pre-emergence damping off, but they also help ensure the development of a healthy root system that accelerates crop growth and establishment, making the plants less vulnerable to infection. Similarly, the rapid emergence and establishment of treated seeds may help improve early canopy closure, which in turn may improve light interception, help shade out weeds, reduce moisture loss, and increase yield.

Fungicides belonging to different chemistry groups that vary in their biochemical mode of action are currently registered for the management of soybean seedling diseases. Depending on the target site, the activity of a fungicide may be directed towards important cellular processes including cell division, respiration, nucleic acid, and protein or RNA synthesis (Fungicide Resistance Action Committee 2016). Four fungicide chemistry groups that currently are used to control RRHR of soybean include the quinone outside inhibitors (QoIs), succinate dehydrogenase inhibitors (SDHIs), demethylation inhibitors (DMIs), and the phenylpyrroles (PP). A partial list of the commonly-used active ingredients within each group includes azoxystrobin, pyraclostrobin, and trifloxystrobin for the QoIs; carboxin, penflufen, and sedaxane for the SDHIs; ipconazole and prothioconazole for the DMIs; and fludioxonil for the PPs. While the QoIs and SDHIs act by inhibiting different mitochondrial respiration pathways in fungi, the

DMIs target C14-demethylase, a critical component of the fungal sterol biosynthesis pathway (Ragsdale 1975; Koller 1988), and the PPs interfere with MAP kinase protein, inhibiting osmotic signal transduction, a process necessary for spore germination and mycelial growth (Mueller and Bradley 2008). Members of these fungicide groups possess a broad spectrum of activity against a broad range of fungal pathogens but are known to act at a single site within a metabolic pathway in the target fungus, thereby increasing the risk of selecting for resistant fungal genotypes. Fungicide resistance is characterized by a reduced sensitivity in the population of a target pathogen to a fungicide (Brent and Hollomon 2007; McGrath 2004) that once provided optimum control of the disease it caused, and resistance is considered to be heritable upon occurrence (Brent and Hollomon 2007). Generally, resistance to fungicides is influenced by factors such as application frequency, the amount of genetic variants within the pathogen population (Hewitt 1998), and the breadth of activity of the fungicide. Fungicides that act at a single site within a metabolic pathway in the fungus generally have a high risk for resistance development since a single mutation is all that is required to allow the fungus to overcome the activity of the fungicide (Hewitt 1998; McGrath 2004). According to the Fungicide Resistance Action Committee (2016), the risk for resistance development to the QoI fungicides is high, and cross-resistance of a fungus will occur across all active ingredients within this class. The SDHIs are classified as medium to high resistance-risk fungicides, the DMIs are considered medium resistance-risk fungicides, while the risk of resistance to the PPs is low to medium. Resistance to the QoIs (Fraaije et al. 2003; Kim et al. 2003; Ma et al. 2003; Pasche et al. 2004; Sierotzki et al. 2005; Wise et al. 2009; Zhang et al. 2012), SDHIs (Avenot and Michailides 2007; Avenot et al. 2012; Gudmestad et al. 2013; Yin et al. 2011), and the DMIs (Bayles et al. 2000; De Waard et al.

1986; Karaoglanidis et al. 2000; Mavroeidi and Shaw 2005; Miller and Gubler 2003; Napier et al. 2000) have been reported in different pathosystems.

Seed treatment fungicide products that generally are utilized by soybean growers in the United States contain combination of active ingredients with multiple modes of action. However, these products may sometimes contain only one fungicide active ingredient that has efficacy against *R. solani*, which may be a single-site inhibitor. The complete reliance on single-site inhibitors seed treatments as a control measure increases the risk for selection of fungicide resistant isolates in the populations of *R. solani* causing seedling disease of soybean. Currently, resistance to the SDHI, DMI, and PP classes of fungicides has not been reported for *R. solani*; however, resistance to QoI fungicides has been identified in AG-1 populations of *R. solani* affecting rice and soybean in the southern United States (Olaya et al. 2012). Certain practices, including the development of fungicide monitoring programs, the adoption of integrated pest management, an avoidance of multiple applications of seed treatments with similar modes of action, and an alternation or combination of fungicides with different modes of action, can help slow down fungicide resistance development in populations of plant pathogenic fungi (Hewitt 1998; Mueller et al. 2013). A fungicide resistance monitoring program, the first and most proactive approach, starts with determining the baseline sensitivity of isolates with no previous exposure to a fungicide group in laboratory conditions, after which, subsequent assays on exposed field isolates can be conducted to determine shifts in sensitivity over time (Mueller et al. 2013).

The sensitivity of *R. solani* to several fungicide classes has been studied in in vitro assays (Amaradasa et al. 2014; Barnes et al. 1990; Blazier and Conway 2004; Campion et al. 2003; Carling et al. 1990; Goll et al. 2014; Kataria et al. 1991; Martin et al. 1984). Moreover, many of

the fungicide tested in in vitro assays, with the exception of few examples like flutolanil and carboxin, currently are not in use for managing either seedling or foliar diseases caused by this pathogen, since fungicides with newer chemistries and systemic properties are now being utilized. Different host crops show susceptibility to a range of AGs, implying that effective chemical control of any given *Rhizoctonia* disease would require fungicides with broad spectrum of activity against potential disease-causing AGs (Kataria and Gisi 1996). Given the ability of different AGs to cause infection on soybean, effective disease control would require an evaluation of the activity of current seed treatment fungicides on all AGs associated with soybean seedling diseases, but such studies have yet to be reported. In in vitro assays, carboxin was shown to have a strong activity towards AG-2 and AG-4 (Kataria et al. 1991; Martin et al. 1984), but not towards AG-3 isolates (Martin et al. 1984). All *Rhizoctonia* spp. recovered from fields in Europe, including AG-3, AG-4, and AG-5 isolates of *R. solani*, were effectively controlled by sedaxane in in vitro assays (Goll et al. 2014). Isolates of AG-2, AG-3, AG-4, and AG-5 showed moderate sensitivity to hexaconazole, while those of AG-7 were highly sensitive (Carling et al. 1990); however, AG-4 isolates recovered from diseased peanut and cowpea plants were highly sensitive to three DMI fungicides (diniconazole, cyproconazole, and tebuconazole) (Barnes et al. 1990). AG-2-2IIIB isolates recovered from creeping bentgrass was highly insensitive to azoxystobin compared to AG-2-2IV isolates from zoysiagrass (Blazier and Conway 2004). Iprodione, a dicarboximide has been shown to effectively inhibit AG-2, AG-3, and AG-4, and AG-5 isolates in vitro (Kataria et al. 1991; Martin et al. 1984; Amaradasa et al. 2014), but Carling et al (1990), reported variability in sensitivity among AG-2-2, AG-3, AG-4, AG-5, and AG-7 isolates. AG-5 isolates from potato in France were highly sensitive to flutolanil, while AG-3 isolates exhibited a range of sensitivity patterns (Campion et al. 2003). More

recently, isolates recovered from soybean were shown to be more sensitive to SDHI than to DMI fungicides (Ajayi and Bradley 2014). The efficacy of fungicide seed treatments on the control of Rhizoctonia seedling disease of soybean has been evaluated both in controlled and field conditions (Bradley 2008; Dorrance et al. 2003; Urrea et al. 2013). In a greenhouse experiment conducted by Dorrance et al. (2003) , none of the fungicide seed treatments evaluated prevented root rot or stand count loss of soybean; however, reduced root rot ratings and higher stand counts were obtained from seed treatments that contained PCNB + thiabendazole or carboxin + imazalil + thiabendazole. From field research trials conducted in North Dakota, Bradley (2008) reported that seed treatments reduced stand and yield reductions when soybeans were planted into cool and moist soil conditions. Urrea et al (2013) found that, compared to the selective fungicides, fungicides with broad spectrum activity increased stand count over a wide range of temperature. Given the ability of *R. solani* to cause infections at varying soil temperature and moisture conditions, as well as the complex interactions with other cool and warm season seedling pathogens, fungicides that offer protection for an extended period during the early stages of seedling growth would be ideal for managing seedling disease of soybean, including Rhizoctonia root and hypocotyl rot; however, the short-term protection offered by most fungicide seed treatment due to the separation of the radicle and hypocotyl from the fungicide remnant on the cotyledon (Dorrance et al. 2003) suggests that an integrated pest management practice that ensures season-long protection should be adopted.

**Future Directions**

Many questions relating to the taxonomy, nomenclature, population biology, and pathogenicity of *R. solani* remain unanswered. Given the complexity surrounding the taxonomy of *Rhizoctonia* and the extent of genetic diversity among isolates classified as *R. solani*, there is

the need for further clarifications on the correct taxonomic relationship among *Rhizoctonia*-like fungi before a consensus can be reached on how these groups of fungi are to be named. Studies expounding the mechanism of variation in this species would be invaluable in providing an in-depth understanding of the roles of sexual recombination and heterokaryosis in creating diversity, and will significantly advance our knowledge in areas relating to its pathogenicity, host specificity, and management. Similarly, an in-depth understanding of the genetic basis of heterogenic incompatibility and the different mating systems within each AG will, no doubt, move us several steps forward in our attempts to clarify the species concept in this species complex. However, given the limitations imposed by sterility of certain isolates (Ogoshi 1987) and the lack of clamp connections to help differentiate between homokaryons and heterokaryons (Cubeta and Vilgalys 1997), other techniques, which likely will include a combination of molecular biology and bioinformatics, would have to be adopted to accomplish this task. Little progress has been made in understanding the molecular mechanisms of the interaction of *R. solani* with different hosts. Information on host-pathogen relationships can enhance the identification and introduction of new resistance in different hosts. Specifically, molecular aspects of the pathogenesis of AG-2-2IIIB and AG-4 on soybean would provide a wealth of information that can be exploited for improving host resistance, the most sustainable management option. With the availability of a reference genome for some AGs (Cubeta et al. 2014; Wibberg et al. 2013), different sequencing platforms can be exploited for pathogenesis and population biology studies, especially for AG-2-2 and other AGs for which our knowledge of population genetic structure and the molecular mechanism of pathogenicity is limited.

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## Tables

**Table 1.1.** Anastomosis groups and subgroups of *Rhizoctonia solani*, host crops, and disease on soybean

| Anastomosis Group (subgroups) | Host Crop | Disease on Soybean | References for AG and Subgroup Designation / References for host crop |
|---|---|---|---|
| **AG-1 (1A, 1B, 1C, 1D, 1E, 1F)** | Rice, Corn, **Soybean**, bean, Turfgrass, Cabbage, Lettuce, Sorghum | Web-blight (Jones and Belmar 1989; Yang et al. 1990a) | Ogoshi 1972; Parmeter et al. 1969; Priyatmojo et al. 2001; Richter and Schneider, 1953; Watanabe and Matsuda 1966; Kuninaga et al. 2002 |
| | | | Grosch et al. 2004; Herr 1992; Jones and Belmar 1989; Martin and Lucas 1984; Mukou et al. 1975; Priyatmojo et al. 2001; Yang et al. 1990a |
| **AG-2- (1, t, Nt ) AG-2- (2IIIB, 2IV, 2LP, 3, 4)** | **Soybean**, Sugar beet, Rice, Turfgrass, Corn, Canola, Wheat, Tulip, Tomato | Seed rot, pre- and post-emergence damping off, Hypocotyl and root rot (AG 2-2IIIB) (Dorrance et al. 2003; Muyolo et al.1993a; Nelson et al. 1996) | Carling et al. 2002; Hyakumachi et al. 1998; Kuninaga et al. 2000; Liu and Sinclair 1991; Naito and Kanematsu 1994; Nicoletti et al. 1999; Ogoshi 1972, 1987; Parmeter et al. 1969; Richter and Schneider 1953; Schneider et al. 1997; Watanabe and Matsuda 1966 |
| | | Foliar blight (AG-2-3) (Naito et al. 1995) | Dorrance et al. 2003; Engelkes and Windels 1996; Liu and Sinclair 1991; Misawa and Kuninaga 2010; Muyolo et al. 1993a; Nelson et al. 1996; Paulitz et al. 2006; Schneider et al. 1997; Sumner and Bell 1982; Watanabe and Matsuda 1966; Windels and Nabben 1989 |

**Table 1.1 (cont.)**

| Anastomosis Group (subgroups) | Host Crop | Disease on Soybean | References for AG and Subgroup Designation<br>References for host crop |
|---|---|---|---|
| **AG-3 (TB, PT, TM)** | Potato, Tobacco, **Soybean**, Tomato | Small lesions on roots (Nelson et al. 1996) | Kuninaga et al. 2007; Kuninaga et al. 2000; Ogoshi 1972; Parmeter et al. 1969; Richter and Schneider 1953; Stevens-Johnk et al. 1993; Watanabe and Matsuda 1966<br>Meyer et al., 1990; Misawa and Kuninaga, 2010; Nelson et al. 1996; Windels et al. 1997; Woodhall et al. 2007 |
| **AG-4 (HGI, HGII, HGIII)** | **Soybean**, Tomato, Dry bean, Peanut, Cotton, Potato, Melon, Broccoli, Spinach | Hypocotyl rot (Bolkan and Ribeiro 1985; Muyolo et al. 1993a; Yang 1999) | Kuninaga and Yokosawa 1984a; Ogoshi, 1972; Parmeter et al. 1969; Richter and Schneider 1953; Steven-Johnk and Jones 2001; Watanabe and Matsuda 1966<br>Balali et al. 1996; Brenneman 1996; Fenille et al. 2002; Kuramae et al. 2003; Muyolo et al. 1993b; Rothrock 1996; Windels and Nabben 1989 |
| **AG-5** | Potato, Turfgrass, Bean, **Soybean**, Sugar beet | Post- emergence damping off (Nelson et al. 1996; Zhao et al. 2005a) | Ogoshi 1972; Richter and Schneider 1953<br>Balali et al. 1995; Martin and Lucas 1984; Nelson et al. 1996; Windels et al. 1997 |

**Table 1.1 (cont.)**

| Anastomosis Group (subgroups) | Host Crop | Disease on Soybean | References for AG and Subgroup Designation<br>References for host crop |
|---|---|---|---|
| **AG-6 (HG-I, GV)** | Non-pathogenic (Mychorrizal with orchids) | None | Kuninaga and Yokosawa 1984b; Shiro Kuninaga, Yokosawa, and Ogoshi 1978<br>Carling et al. 1999; Pope and Carter 2001 |
| **AG-7** | **Soybean**, Potato, Cotton, Water melon | | Homma et al. 1983<br>Abd-Elsalam et al. 2010; Baird et al. 1996; Carling et al. 1998 |
| **AG-8-ZG (1, 2, 4, 5)** | Small grains (Wheat, Barley, e.t.c) | None | MacNish and Sweetingham 1993; Neate and Warcup 1985<br>Neate and Warcup 1985; Roberts and Sivasithamparam 1986 |
| **AG-9 (TP, TX)** | Potato, lettuce, carrot | None | Carling and Kuninaga 1990; Carling et al. 1987<br>Carling et al. 1987 |

**Table 1.1 (cont.)**

| Anastomosis Group (subgroups) | Host Crop | Disease on Soybean | References for AG and Subgroup Designation<br>References for host crop |
|---|---|---|---|
| **AG-10** | Non-pathogenic | | MacNish et al. 1995 |
| **AG-11** | Wheat, Lupin, **Soybean**, Cotton, Potato, Radish | | Carling et al. 1994<br>Carling et al. 1994; Kumar et al.1999; Sweetingham 1989 |
| **AG-12** | Cauliflower, Radish, Mychorrizal with orchids | | Carling et al. 1999<br>Carling et al. 1999; Pope and Carter 2001 |
| **AG-13** | Non-pathogenic | | Carling et al. 2002 |
| **AG-BI (Bridging Isolate)** | Non-pathogenic | | Kuninaga et al. 1978 |

**Table 1.2.** Model for pathogenesis in *Rhizoctonia solani* and putative pathogenesis-related genes (Rioux et al. 2011)

| Phase | Putative pathogenesis-related genes | Function |
|---|---|---|
| Host contact and penetration | Polysaccharide synthase/glycosyltransferase family | Contact and appressorium formation |
| | RAB GTPase | Secretion of cell wall degrading enzyme and vesicular trafficking |
| Adjustment to host environment | Glutathione-*s*-transferase kappa 1 | Inactivation and degradation of toxic compounds |
| | Pyruvate carboxylase | Metabolism and Gluconeogenesis |
| | ABC transporter | Efflux and protection against plant defenses |
| Growth in necrotic plant tissue | Glutathione-*s*-transferase kappa 1 | Inactivation and degradation of toxic compounds |
| | Pyruvate carboxylase | Metabolism and Gluconeogenesis |
| | Major facilitator superfamily multidrug - DHA1 subfamily protein | Toxin secretion; efflux; protection against plant defense |

## Figures

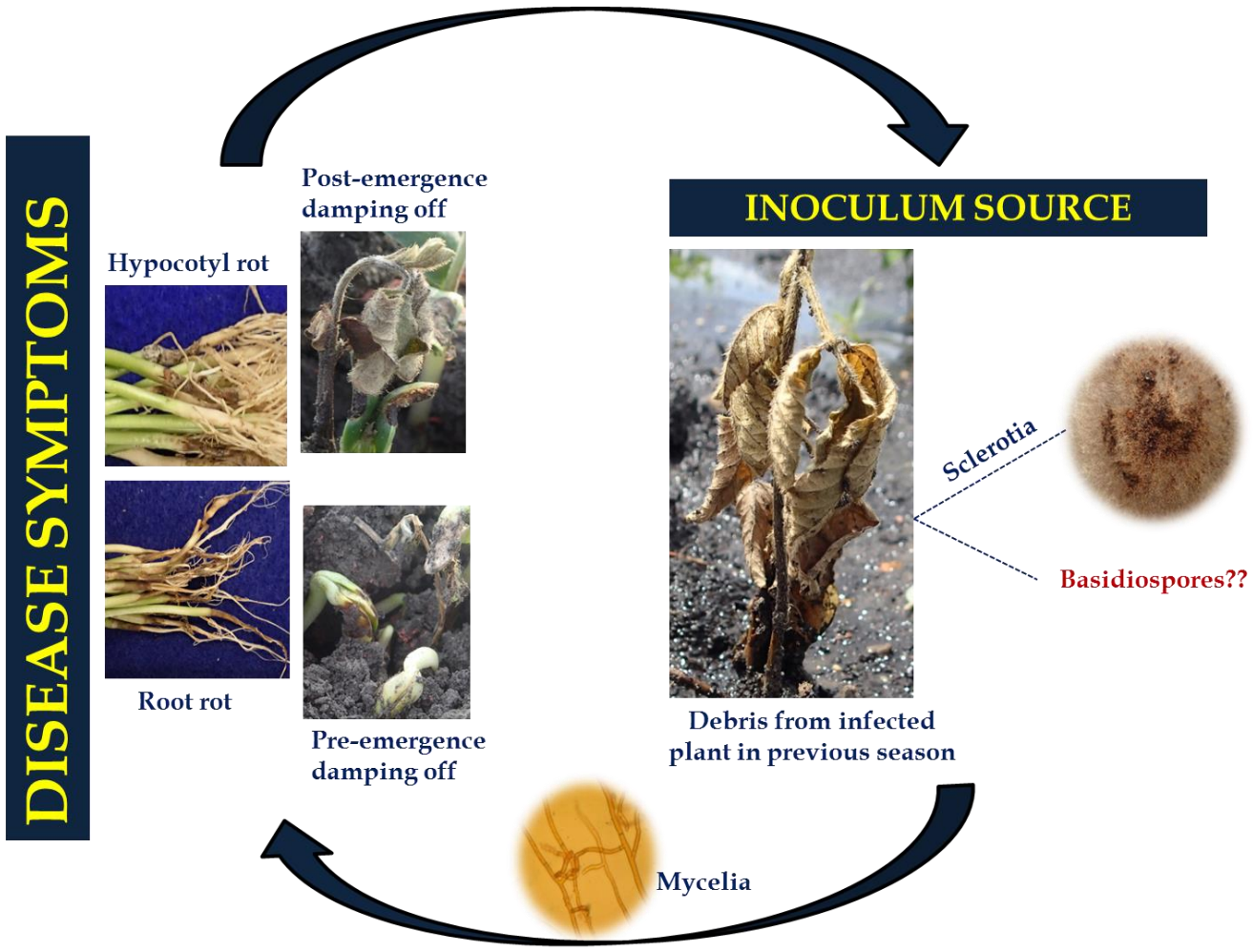


**Fig 1.1.** Disease cycle of *Rhizoctonia solani* causing seedling disease on soybean

# CHAPTER 2: IDENTIFICATION AND CHARACTERIZATION OF *RHIZOCTONIA* SPECIES ASSOCIATED WITH SOYBEAN SEEDLING DISEASE


## Abstract

In an effort to identify the important species of *Rhizoctonia* associated with seedling diseases of soybean, isolates of *Rhizoctonia* spp. were recovered from soybean seedlings with damping off and root and hypocotyl rot symptoms from Arkansas, Illinois, Kansas, Michigan, Minnesota, and the Canadian province of Ontario between 2012 and 2014. Based on morphology, PCR-RFLP and phylogenetic analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA genes, 79 isolates were confirmed to be *R. solani*, 24 were binucleate *Rhizoctonia* spp., and 10 were *R. zeae*. Of the 79 *R. solani* isolates, 51 belonged to anastomosis group (AG) 2-2IIIB, five belonged to AG-3 PT, three belonged to AG-4 HGI, two belonged to AG-4 HGIII, nine belonged to AG-7, and eight belonged to AG-11. Bayesian inference of phylogeny using the ITS region revealed two clusters of *R. solani* AG-7 that possibly correspond to different AG-7 subgroups. Phylogenetic analysis also provided evidence for genetic relatedness between certain binucleate *Rhizoctonia* and some *R. solani* isolates. On soybean cultivar 'Williams 82', isolates of AG-2-2IIIB were the most aggressive, followed by isolates of AG-7, AG-4, and AG-11. On 'Jubilee', a sweet corn cultivar, AG-2-2IIIB and AG-4 isolates caused significant stunting and root damage, while the damage caused by the AG-11 isolates was mostly restricted to the mesocotyl. Isolates of *R. zeae* and the binucleate *Rhizoctonia* spp. were not pathogenic on soybean or corn. Our results indicate that soybean and corn are hosts to the predominant and aggressive AGs of *R. solani*, implying that rotation between these two crops may not be an effective management practice.

## Introduction

Seedling diseases have a history as a major constraint to soybean (*Glycine max* (L.) Merr.) production in North America. In the north central U.S., the estimated impact of seedling diseases on soybean yields was noted between 1989 to 1991 (Doupnik 1993), and between 2003 and 2005, a nation-wide estimated suppression in yield was reported, with the greatest impact occurring in Illinois, Ohio, Minnesota, Kansas, and North Dakota (Wrather and Koenning 2006). From 2006 to 2009, seedling diseases ranked third among diseases and pathogens that reduced soybean yields, behind only Phytophthora root and stem rot and soybean cyst nematode (Koenning and Wrather 2010). In 2006, both early and late season estimated yield losses were attributed to soybean cyst nematode and seedling diseases in Canada (Wrather et al. 2010).

Among the myriad of plant pathogens associated with the seedling disease complex of soybean, fungi classified as members of the species *Rhizoctonia solani* (Kuhn) (syn. *Thanatephorus cucumeris* (A. B. Frank) Donk) represent an important and intractable group. Besides having a necrotrophic and saprophytic lifestyle, this group of plant pathogens exhibit significant genetic diversity often evident as differences in colony morphology, biochemical and molecular properties, and pathogenicity on different hosts. As a consequence, members of this species complex have been classified into fourteen anastomosis groups (AG) (AGs 1 to13, and AG-BI) (Carling et al. 1999; Carling 1996; Ogoshi 1987), which are considered to be non-interbreeding populations (Anderson 1984) with distinct evolutionary origins (Vilgalys and Cubeta 1994). On the basis of characteristics other than hyphal fusion, several AGs have been further divided into subgroups, and a few of these AGs and subgroups have been associated with seedling disease of soybean. Around the globe, isolates of the subgroup AG-2-2IIIB (Fenille et al. 2002; Liu and Sinclair 1991; Muyolo et al. 1993; Sneh et al. 1991) and of those of AG-4

(Bolkan and Ribeiro 1985; Fenille et al. 2002; Muyolo et al. 1993; Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996; Zhao et al. 2005) and AG-5 (Nelson et al. 1996) have been identified as the major culprits causing seedling diseases of soybean. Other less aggressive AGs associated with soybean seedling diseases include AG-3 (Nelson et al. 1996), AG-7 (Baird et al. 1996; Rothrock et al. 1993), and AG-11 (Carling et al. 1994).

In the United States, Rhizoctonia root and hypocotyl rot is an important seedling disease of soybean, especially in the north central region (Doupnik 1993), where more than 80% of the total U.S. soybean production occurs (American Soybean Association 2015). Reports of Rhizoctonia root rot epidemics on soybean first emerged during the 1967 growing season in Iowa, where a reduction in stand was observed (Tachibana 1968). In 1971, as high as a 48% reduction in yield was reported in small research plots (Tachibana et al. 1971). Causal AGs or species of *Rhizoctonia* were unknown at the time the epidemic occurred; however, in 1996, Rizvi and Yang (1996) identified AG-2-2 and AG-4 isolates as the primary AGs of *R. solani* associated with soybean seedling disease in Iowa.

Very limited efforts have been directed towards the identification and characterization of *Rhizoctonia* species associated with soybean seedling disease in other soybean growing regions of North America. Besides Iowa, a survey of the prevalent species of *Rhizoctonia* that attack soybean has been conducted in Ohio (Muyolo et al. 1993), the Red River Valley of Minnesota and North Dakota (Nelson et al. 1996), and the Canadian province of Ontario (Zhao et al. 2005). Given that the population of *R. solani* affecting soybean seedlings in other major soybean-producing states in the U.S. has not been extensively investigated, information regarding the pathogen variability and distribution across the country is scarce. Cropping systems differ across soybean growing locations, and these differences can significantly enhance the prevalence of

certain species of *Rhizoctonia* over others. Moreover, *R. solani* AGs and subgroups differ not only in their aggressiveness on specific host crops, but also in their sensitivity to different fungicide seed treatments (Kataria et al. 1991; Martin et al. 1984a; Carling et al. 1990; Barnes et al. 1990). Therefore, information on the predominant groups, together with knowledge of their pathogenic potential is needed for making sound disease management decisions.

Illinois is a leading producer of soybean in the U.S.; unfortunately, a state-wide investigation of the predominant *Rhizoctonia* species causing soybean seedling diseases has not been carried out to date. In the early 1990s, two highly aggressive AG 2-2 isolates, 61D-3 and 65L-2, were recovered from diseased soybean plants collected from a soybean breeding plot at the University of Illinois (Liu and Sinclair 1991), but nothing is known about the variability in the AGs associated with Rhizoctonia root and hypocotyl rot of soybean in the state. Similarly, the *Rhizoctonia* species associated with soybean seedling diseases in Arkansas, Michigan, Minnesota, and, Kansas are unknown. Therefore, the objectives of this study were to (i) identify and characterize the species of *Rhizoctonia* associated with soybean seedling disease in Illinois, Arkansas, Michigan, Minnesota, and Kansas (ii) determine if there has been a change in the AGs of *R. solani* that damage soybean seedlings in Ontario, Canada, and (iii) determine the pathogenicity of the isolates recovered from Illinois on soybean and corn.

## Materials and Methods

### Isolate collection and storage

Soybean seedlings showing hypocotyl and root rot symptoms typical of *R. solani* infections were collected from thirteen Illinois counties in 2012, 2013, and 2014 (Table 2.1). Plants were dug carefully to minimize root damage, then placed in sealed plastic bags, and placed in a cooler during transport back to the lab, where they were stored overnight at 4°C. Roots of plants were

washed under running tap water to remove soil particles. Plants were then disinfected in 1% NaOCl solution for 30 s, followed by a thorough rinse in distilled water for 1 min. Cut sections of about 3 mm in length from symptomatic plants were gently pressed onto solidified Ko and Hora medium (Ko and Hora 1971) and incubated in the dark at 25°C for 24 to 48 h. Hyphal tips from colonies with right-angled branches typical of *Rhizoctonia*-like fungi were transferred to potato dextrose agar (PDA; Becton, Dickson and Company, Sparks, MD) amended with 25 mg/liter of rifampicin and were incubated at 25°C. Isolates recovered from Arkansas, Kansas, Michigan, Minnesota, and Ontario were collected as part of a multi-state research project by the laboratories of Dr. Craig Rothrock (University of Arkansas, Fayetteville), Dr. Christoper Little (Kansas State University, Manhattan), Dr. Martin Chilvers (Michigan State University, East Lansing), Dr. Dean Malvick (University of Minnesota, St. Paul), and Mr. Albert Tenuta (Ontario Ministry of Agriculture and Rural Affairs, Ridgetown), respectively. These isolates were then sent to Dr. Ahmad Fakhoury's laboratory (Southern Illinois University, Carbondale), where they will continue to be curated as part of the multi-state research project. Tester isolates of *R. solani* used for comparative purposes and their respective origins are listed in Table 2.2. All isolates were maintained on sterile table beet seeds. These were dried under a sterile flow hood for 48 h, placed in 2.0 ml Nalgene cryogenic vials (Sigma-Aldrich, St. Louis, MO), and stored at 4°C. For long-term storage, three plugs (5 mm diameter) of isolates on PDA were placed in 1.5 ml micro centrifuge tubes containing 850 µl of 15% glycerol and maintained at -80°C.

**Morphological characterization and anastomosis group-typing of isolates**

All isolates were characterized based on cultural morphology, cellular nuclei number (CNN) in young vegetative hyphae, and anastomosis reactions with tester isolates. Cultural characteristics were determined by growing each isolate on PDA and incubating at 25°C for 14

days. Isolates identified as members of AG-2-2 were further maintained at 35°C to enable rapid delineation of the AG-2-2IIIB isolates (Sneh et al. 1991). Cultures were examined for colony color, sclerotia formation and color, aerial mycelia formation, and growth zonation at 5 and 14 days after plating. Binucleate *Rhizoctonia* (BR) were differentiated from multinucleate *Rhizoctonia* by an examination of the nuclear condition using the Safranin O staining technique (Bandoni 1979; Yamamoto and Uchida 1982). For AG determination, a modified version of the clean slide technique (Kronland and Stanghellini 1988) coupled with Safranin O staining was used for observing hyphal fusion reactions between testers and unknown isolates. Briefly, the unknown isolates and testers initially were grown on PDA for 3 to 5 days. Plugs (5 mm in diameter) of the unknown isolate were cut from the growing edge of the plate and transferred to the opposite ends of a 100 mm x 15 mm petri dish containing 1.5% water agar (WA). A 5 mm plug of the tester isolate was placed about 4 cm away from the unknown isolate and incubated at 25°C for 24 to 72 h, depending on the growth of the interacting isolates. Cut sections of overlapping hyphae were placed on a plain microscope glass slide, stained with safranin O and 3% KOH, and covered with a coverslip before viewing under a compound microscope. Reaction types observed between interacting hyphae were assigned to one of the four categories, C0, C1, C2 or C3, as described by Carling et al. (1988). Pairing for each unknown isolate was replicated twice, and at least four anastomosing points were required for a definitive placement of an unknown isolate in an AG. Pictures of cellular nuclear number and anastomosis reaction types were taken with a Zeiss Axiocam ERc5s and viewed via a Zeiss ZEN imaging software attached to a PC.

**DNA isolation, RFLP-PCR analysis of the rDNA-ITS regions, and PCR analysis with AG-specific primers for AG-typing of *R. solani* isolates**

For genomic DNA extraction, each isolate was grown on potato dextrose broth (Becton, Dickson and Company, Sparks, MD) and incubated in continuous light without shaking at 25°C. Mycelia were harvested after 14 days, placed in 1.5 ml micro centrifuge tubes, and stored at -20°C. DNA extraction was carried out using the FastDNA® spin kit (MP Biomedicals, Santa Ana, CA) following the manufacturer's recommendations for fungal DNA isolation. Not all isolates identified as *R. solani* could be classified to AG using the traditional hyphal reaction technique; therefore, a PCR-RFLP analysis of the internal transcribed spacer (ITS) regions of the ribosomal genes was initially conducted on all tester isolates and then on the recovered *R. solani* isolates to allow for a comparison of restriction patterns and final AG-typing. For this analysis, genomic DNA was amplified using primer pairs, RS1 (5′-CCTGTGCACCTGTGAGACAG-3′) and RS4 (5′-TGTCCAAGTCAATGGACTAT-3′) (Guillemaut et al. 2003). PCR reactions were performed in a 40 µl mixture containing 20 µl of GoTaq® Green Master Mix 2X (Promega Corporation, Madison, WI), 4 µl each of RS1 and RS4 primers, 4 µl of nuclease-free water, and 8 µl of template DNA. PCR amplification was done in a 2720 thermal cycler (Applied Biosystems, Foster City, CA) using the following cycle parameters: initial denaturation at 94°C for 3 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Restriction analysis of PCR products was carried out in separate digestion reactions with the enzymes *Mse*I (Fisher Scientific), *Mun*I (Fisher Scientific), *Ava*II (Promega Corporation, Madison, WI), and *Hinc*II (Promega Corporation, Madison, WI) following manufacturer's protocols. *Ava*II and *Hinc*II were combined in a single reaction for double digestion. Aliquots (5 µl) of restriction fragments were

checked on 2.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC) and viewed with GelCam 310 Camera attached to a GelDoc-It$^2$ Imager (UVP, LLC, Upland, CA). Restriction patterns of the unknown isolates and tester isolates were compared with those reported by Guillemaut et al. (2003) for correct placement of isolates into AGs and subgroups. The rDNA-ITS region of the AG-2-2IIIB isolates were amplified using AG-2-2IIIB-specific primers (Carling et al. 2002), while AG-3 isolates were amplified using both AG-3 (Lees et al. 2002) and AG-3 subgroup-specific primers (Kuninaga et al. 2000). Forward and reverse primers for the AG-2-2IIIB isolates were 5′-AGGCAGAG(A/G)CATGGATGGGAG-3′and 5′-ACCTTGGCCA(A/C)CCTTTTTATC-3′), respectively. For the AG-3 isolates, AG-3-specific primer pairs used were Rs1F2 (5′-TTGGTTGTAGCTGGTCTATTT-3′) and Rs2R1 (5′-TATCACGCTGAGTGGAACCA-3′), while the AG-3 subgroup-specific primer pair was (5′-GTTTGGTTGTAGCTGGCCC-3′) and (5′-CTGAGATCCAGCTAATGT-3′) for the AG-3TB (tobacco type) and (5′-GTTTGGTTGTAGCTGGTCT-3′) and (5′-CTGAGATCCAGCTAATAC-3′) for the AG-3PT (potato type).

**PCR amplification of rDNA-ITS regions, sequencing, and phylogenetic analysis**

The rDNA-ITS regions of 57 collected and 16 tester isolates (Table 2.1) were amplified with the ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-5 primers (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990) in a final volume of 40 µl using the PCR reactions and cycle parameters described above. Successful amplification was confirmed by analyzing aliquots (5 µl) of PCR products on 1% agarose gel and viewing with a GelCam 310 Camera attached to a GelDoc-It$^2$ Imager (UVP, LLC, Upland, CA). The PCR product for each isolate was purified with the Wizard® SV Gel and PCR Clean-Up System

(Promega Corporation, Madison, WI) and sequenced at the McLab Molecular Cloning Laboratories (South San Francisco, CA) with the ITS-4 and ITS-5 primers. ITS sequences for both complimentary strands for each isolate were manually edited and assembled to produce a consensus sequence using the software Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI). Reference sequences representative of the AGs of *R. solani* as well as those of other species of *Rhizoctonia* were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Table 2.3) for comparative purposes. To ascertain that all unknown isolates belonged to the genus *Rhizoctonia*, the consensus sequence of each isolate was queried using the BLASTN option (Altschul et al. 1997) in the NCBI database. Consensus sequence alignment for the unknown isolates (57), tester isolates (16), and those of the reference isolates (24) was conducted using the ClustalW program in the Molecular Evolutionary Genetics Analysis (MEGA version 6) Software (Tamura et al. 2013). Best-fit substitution model selection for phylogenetic analyses of the aligned consensus sequences was carried out using the Modeltest 3.7 program (Posada and Crandall 1998) implemented in PAUP* (Swofford 1998), with model selection strictly on the basis of the Akaike Information Criterion (AIC) estimate (Akaike 1974). Based on the AIC, the transversion model plus gamma (TVM+G) was selected by Modeltest 3.7, with the following parameters: number of substitution types = 6; rates at variable sites = gamma; state frequencies for A, C, G, and T = 0.33, 0.20, 0.20, and 0.27, respectively; substitution rates for the TVM rate matrix [AC, AG, AT, CG, CT,GT] = 0.75, 2.71, 1.07, 0.68, 2.71, and 1.00; shape parameter of the gamma distribution of rate variation = 0.37; proportion of invariable sites = 0. A phylogenetic tree was then computed from the selected model and parameters using a Bayesian Metropolis-coupled Markov Chain Monte Carlo analysis implemented in MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003). The MCMC analysis was run

for 2 million generations with chain sampling every 500 generations. From the pool of trees constructed after convergence, 50% majority rule trees were constructed. Bayesian posterior probability (PP) or clade support values were estimated from the consensus tree, and nodes with PP ≥ 95% were considered strongly supported. Isolates were assigned to a species, AG, or subgroup if they clustered with a reference isolate in a strongly-supported clade. Phylogenetic trees were visualized using the FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

**Greenhouse pathogenicity assay**

For greenhouse pathogenicity tests on soybean and corn, we selected representative isolates from the Illinois collection. Forty eight (Table 2.4) and sixteen (Table 2.5) isolates representative of each of the identified AGs and the other species of *Rhizoctonia* were evaluated in the greenhouse for their ability to cause hypocotyl and root rot symptoms on soybean and corn, respectively. Since isolates of AG-2-2IIIB were the most frequently recovered, the number of isolates within this group that was evaluated for pathogenicity was greater than those of other groups. This permitted an evaluation of the variability in aggressiveness of members of this group. Inoculum preparation was similar to that described by Paulitz and Schroeder (2005) but with slight modifications. Briefly, sorghum seeds were autoclaved for 30 min at 20 psi and at 120°C. A second autoclaving was carried out after 24 h using the same parameters described for the first autoclaving. Plugs (5 mm) from 4-day old cultures of each isolate were transferred to separate 250 ml flasks containing autoclaved sorghum seeds and incubated in the dark at 25°C for two weeks. Each flask was shaken at least twice per week to ensure uniform colonization of seeds. After two weeks of incubation, seeds were dried under a laminar flow hood for 3 days, packaged in paper bags, and stored at 4°C before use. A modified version of the inoculum-layer technique (Schmitthenner and Hilty 1962) was adopted for inoculation. Ten non-treated seeds of

soybean cv. Williams 82 were sown into 15 cm diameter plastic pots that had been previously half-filled with steam-pasteurized 2:1 sand to silt loam soil, covered with 5 g of inoculum, and covered with a 5 cm layer of soil. Control pots received non-infested autoclaved sorghum seeds. The experiment was arranged as a completely randomized design with three replicates of each experimental unit (pots). The plants were grown in a greenhouse maintained at 24 ± 3°C and a 14 h photoperiod. For corn, 20 cm diameter pots were filled using the inoculum-layer technique described for soybean, and 10 seeds of non-treated sweet corn cv. Jubilee was sown directly over the uppermost soil layer. At 21 days after planting, plants were collected, roots were washed under a high pressure flow nozzle, and plants were evaluated for disease severity on hypocotyls and roots. Roots were then dried for 4 d at 50°C and weighed. Disease severity was rated on a 0 to 5 scale modified from Nelson et al. (1996) as follows: 0 = no lesion on root or hypocotyl; 1 = lesions < 2.5 mm on hypocotyl and ≤ 20% of roots with lesions or rot symptoms; 2 = lesions 2.5 to 5 mm on hypocotyl and 20-40% of roots with lesions or rot symptoms; 3 = lesions > 5 mm on hypocotyl and 40-60% of roots with lesions or rot symptoms; 4 = lesions girdling entire hypocotyl and 60-80% of roots with lesions or rot symptoms; and 5 = plant dead, and/or no roots, or > 80% of roots with lesions or rot symptoms. Statistical analyses of dried root weight data were carried out in SAS (version 9.4; SAS institute Inc., Cary, NC). For dry root weight data, normality and homogeneity of residuals were checked using the UNIVARIATE procedure and the Brown and Forsythe's HOVTEST option in SAS, respectively, and data were transformed when necessary. Disease severity data were subjected to nonparametric analysis using the rank-based method described by Shah and Madden (2004). Briefly, median ratings of the 10 plants constituting an experimental unit were obtained before further analysis. Ordinal ratings were converted to midranks using PROC RANK, and midranks obtained were then used

by PROC MIXED to calculate significance levels and nonparametric test statistics. Relative treatment effect ( $\hat{p}_{ij}$ ) of each individual isolate and confidence intervals (CI) of relative treatment effects were obtained using the LD_CI macro written by Brunner et al. (2002) and available at http://www.ams.med.uni-goettingen.de/sasmakr-de.shtml. Isolates were grouped by AG, and contrast statements were used to determine significant differences (at $\propto = 0.05$) among AGs in their ability to cause disease.

## Results

### Morphological characterization

Of the 113 isolates recovered from Illinois, Arkansas, Kansas, Michigan, Minnesota, and Ontario, 79 were identified as *R. solani*, 10 as *R. zeae*, and 24 as BR (*Ceratobasidium* spp.). The 79 *R. solani* isolates were characterized as belonging to five AGs as follows: AG-2 (with two subgroups, AG-2-1 (1) and AG-2-2IIIB (51)), AG-3 (5), AG-4 (5), AG-7 (9), and AG-11 (8). All *R. solani* isolates exhibited macroscopic and microscopic characteristics typical of members of the species (Figs. 2.1a, 2.1d, 2.3); On PDA, young vegetative hyphae were hyaline when young but became brown with age, and hyphal cells were multinucleate. Cellular nuclear number of young vegetative hyphae ranged from 4 to 12 nuclei, with considerable variation among AGs (Table 2.6). After 2 weeks of incubation, the AG-2-1 isolate produced thick-walled, brown sclerotia that darkened with prolonged incubation. Isolates of AG-2-2IIIB exhibited marked similarity in cultural appearance; colonies appeared brown with age with zonation, and the thick-walled, brown sclerotia produced darkened with prolonged incubation. All AG-2-2IIIB isolates grew at 35°C. The AG-2-1 isolate produced a C2 reaction with the AG-2-1 tester isolate Rh051307, while the 10 AG-2-2IIIB selected for anastomosis reaction assays produced a C2 reaction with the AG-2-2IIIB tester isolate 65L-2 (BF09476).

Cultures of AG-3 isolates were buff in color, with no zonation or apparent pigmentation. Also, sclerotia, which were seldom produced, appeared embedded in PDA around the edges of the plate. Anastomosis reactions between the recovered AG-3 isolates and the AG-3 tester isolate, USA-AG3, produced a C2 reaction type. The five AG-4 isolates produced brown colonies on PDA with no zonation or apparent pigmentation. Occasionally, a few olive brown sclerotia developed around the inoculation point on PDA. There were no differences in growth among the four isolates at 25°C, and all four isolates produced a C3 reaction type when anastomosed with AG-4 tester isolate Rh051339. Traditional anastomosis analysis using AG-4 subgroup specific tester isolates did not permit classification of the AG-4 isolates into subgroups. The AG-7 isolates exhibited similarity in cultural morphology to the AG-4 isolates; colony appeared brown at 2 weeks after incubation with clusters of sclerotia occurring mostly at the point of inoculation in PDA. None of the AG-7 isolates anastomosed with any of the AG-4 or AG-7 tester isolates. The AG-11 isolates produced light brown mycelia on PDA at 5 days after incubation at 25°C, but colony color became dark brown with age. Two of the AG-11 isolates produced yellow pigmentation on PDA. Abundant sclerotia, ranging from light tan to brown, were densely distributed on the surface of the PDA. All AG-11 isolates produced a C2 reaction type when anastomosed with AG-11 tester isolate HPIN22A. For all BR, two nuclei per cell were observed in young vegetative hyphae, and no sclerotia were observed in culture after 2 weeks of incubation. Isolates identified as *R. zeae* had multinucleate cell compartments with 4 to 7 nuclei and produced salmon-orange colonies on PDA. In addition, white spherical sclerotia formed, which turned orange to red with age and appeared submerged in PDA.

### *Rhizoctonia* species by location

Of the total number of isolates characterized in this study, 77 were recovered from Illinois, 10 from Arkansas, one from Kansas, one from Michigan, one from Minnesota, and 23 from Ontario, Canada. The identity of the Illinois isolates are as follows: AG-2-2IIIB (28), AG-3 (5), AG-4 (5), AG-7 (4), AG-11 (6), BR or *Ceratobasidium* spp. (19), and *R. zeae* (10). Six of the Arkansas isolates were AG-7, two were AG-11, one was AG-2-1, and two were BR. The Kansas, Michigan, and Minnesota isolates were BR, while all 23 isolates from Ontario were AG-2-2-IIIB.

### RFLP-PCR analysis of the rDNA-ITS regions and PCR analysis with AG-specific primers for AG-typing of *R. solani* isolates

Restriction patterns obtained from the digestion of *R. solani* isolates with the restriction enzymes *Mse*I, *Mun*I, *Ava*II, and *Hinc*II corresponded well with those obtained by Guillemaut et al. (2003). Of the different RFLP types they reported for AG-2-1, only "DANN" was observed for both the AG-2-1 isolate from Arkansas and the AG-2-1 tester isolates Rh051307 and Rh051324. All of the AG-2-2IIIB isolates characterized by restriction analysis produced a "CAAN" RFLP type. None of the four AG-3 isolates could be typed to either AG or subgroup by restriction analysis. Restriction analysis permitted the classification of all AG-4 isolates into subgroups, producing "IEAA", and "LENA" RFLP types for the AG-4 HGI and AG-4 HGIII isolates, respectively. Two RFLP types were observed for the AG-7 isolates; AG-7 tester isolate ST81548 produced "DNNN", while the AG-7 isolates from Arkansas produced an "ENNA". The AG-7 isolates from Illinois could not be resolved by restriction analysis. All AG-11 isolates produced a "BNAA" RFLP type. Isolates identified as AG-2-2IIIB were successfully amplified with the AG-2-2IIIB-specific primers, producing a single amplicon of 0.50 kb. The

AG-3PT primer pair gave a single product of approximately 0.5 kb for the AG-3 isolates recovered from Illinois, while the AG-3TB primer produced a single amplicon of about 0.5 kb for the AG-3 tester isolate USA-AG3.

**Phylogenetic analysis of rDNA-ITS region**

Phylogenetic analysis of the rDNA-ITS sequence data permitted the classification of the recovered isolates into well-established AG or subgroups of different *Rhizoctonia* species. Of the 57 isolates analyzed for phylogenetic relationship with reference isolates from the GeneBank, 56 were identified based on the posterior probabilities (PP) obtained from the Bayesian analysis (Fig. 2.4). Three isolates clustered and formed a strongly-supported clade (PP = 100%) with tester isolate R63_42A and with a *R. zeae* accession from Japan (AB213594). Sixteen isolates were identified as BR and were located in two distinct clades. In the first clade, which consisted mainly of BR isolates, one isolate clustered with the *Ceratobasidium* sp. AG-L reference isolates from Japan (AB286933) (PP = 100%), while 12 isolates clustered with a *Ceratobasidium* sp. AG-A reference isolate from Japan (AF354092) (PP = 100%). The remaining three BR were found to group in a clade consisting of *R. solani* isolates. While two of these isolates grouped closely with two *Ceratobasidium* sp. AG-F reference isolates (PP = 100%) in a cluster comprising AG-4 and AG-7 isolates, one clustered with both AG-6 tester isolate and an AG-6 reference isolate from Japan (AF354102). Two *R. solani* AG-7 clusters were observed from our analysis. The first cluster (PP = 100%) comprised the AG-7 tester isolate ST81548 and a reference AG-7 isolate AF354098 from Japan, while the second cluster (PP = 100%) included nine recovered isolates (four from Illinois and four from Arkansas) and a *R. solani* AG-7 reference isolate from Arkansas (AF153793). Two isolates grouped with *R. solani* AG-4 HGI reference isolate AB000007 from Japan (PP = 100%) and two others clustered with

AG-4 pathogenic tester isolate and *R. solani* AG-4 HGIII reference isolate AY154659 from Brazil (PP = 100%). One recovered isolate clustered with a *R. solani* AG-2-1 reference isolate AY154317 from Brazil with a PP of 98%. Thirteen recovered isolates and two tester isolates, 65L-2 (BF09476) and AG-2-2_Nelson, clustered together and formed strongly-supported clades with two *R. solani* AG-2-2IIIB reference isolates, AF354116 and GU811670 from Japan and the U.S., respectively. Seven isolates were identified as *R. solani* AG-11 based on a PP value of 100% from a cluster formed with tester isolate HPIN22A and an AG-11 reference isolate AF354114 from the U.S. Five isolates formed a strongly supported clade (PP = 100%) with USA_AG-3 tester isolate and an AG-3 reference isolate from the U.S. (GQ885147).

**Greenhouse pathogenicity assay**

**Pathogenicity on soybean.** Forty-eight isolates were evaluated for their ability to incite disease on the roots and hypocotyls of soybean in the greenhouse. Of these, 23 were AG-2-2IIIB, five were AG-3, five were AG-4, four were AG-7, three were AG-11, five were BR, and three were *R. zeae*. Based on disease ratings, RE, and mean ranks, isolates of AG-2-2IIIB were the most aggressive on the roots and hypocotyls of soybean followed by those of AG-7, AG-4, AG-11, AG-3, BR, and *R. zeae* (Table 2.4). The median disease severity ratings of the 48 isolates evaluated ranged from 0.25 to 5, with relative treatment effects ranging from 0.13 to 0.85. Isolates of AG-2-2IIIB had the greatest median disease severity rating (ranging from 2.5 to 5), $\hat{p}_{ij}$ (ranging from 0.50 to 0.85), and mean ranks (ranging from 178.4 to 272), with the exception of two isolates that had median disease severity ratings of 0.75 and 1 and significantly lower $\hat{p}_{ij}$ (0.18 and 0.25, respectively) and mean ranks (58.2 and 84.4, respectively). Isolates of other AGs of *R. solani*, the BR, and *R. zeae* gave median disease ratings less than 2.0 and $\hat{p}_{ij}$ less than 0.50. When isolates were grouped by AG of *R. solani* or species of *Rhizoctonia* and compared

based on $\hat{p}_{ij}$ and their 95% CI, there were significant differences among the different groups in their ability to cause disease, with AG-2-2IIIB causing the greatest damage and the BR and *R. zeae* causing the least damage (Table 2.7). Compared to the non-inoculated control, all isolates significantly reduced dry root weight; however, isolates of AG-2-2IIIB caused the greatest root weight reduction (Fig. 2.5).

**Pathogenicity on corn.** Three isolates each of AG-2-2IIIB, AG-3, AG-11, two isolates each of AG-4, BR, *R. zeae*, and one isolate of AG-7 were evaluated for their ability to incite disease on roots of corn. The median disease ratings ranged from 0 to 5, with $\hat{p}_{ij}$ ranging from 0.25 to 0.92 and mean ranks ranging from 24.5 to 94.3 (Table 2.5). AG-2-2IIIB isolates produced the greatest median disease severity ratings (ranging from 2.5 to 4.5) while isolates of BR gave zero as the median disease severity rating. Based on $\hat{p}_{ij}$ and their 95% CI, AG-2-2IIIB isolates were the most aggressive, although the AG-4 isolates expressed similar levels of aggressiveness (Table 2.8). Isolates of AG-3, AG-7, BR, and *R. zeae* were not significantly different from the non-inoculated control.

**Discussion**

In this study, isolates of *Rhizoctonia* recovered from symptomatic soybean plants between 2012 and 2014 from five states in the U.S. and from the Canadian province of Ontario were characterized and identified to AGs, subgroups, and species. The pathogenic potential of isolates representative of the identified groups was also determined to provide information on which of the groups present the greatest risk to soybean health in any soybean production field. Three taxonomic groups, *R. solani* (70%), *R. zeae* (9%), and *Ceratobasidium* spp. (21%) were identified based on morphological characters and cellular nuclear number. Combining both classical and molecular techniques, isolates of *R. solani* were further identified to AG/subgroups.

Although traditional anastomosis reactions did permit the classification of *R. solani* isolates, certain isolates did not anastomose with tester isolates and could not be identified to an AG or subgroup. Failure of anastomosis has been linked to factors such as nutrient conditions, mutation, and aging, and some isolates may have lost the ability for anastomosis (Carling 1996; Hyakumachi and Ui 1987). In recent years, molecular methods have replaced traditional anastomosis reaction assays for the correct placement of an unknown isolate in an AG or subgroup. To circumvent the limitations of traditional AG-typing, we employed a method involving restriction analysis of the PCR-amplified ribosomal DNA (Guillemaut et al. 2003) to characterize the *R. solani* isolates recovered in this study. Isolates of the different AGs were differentiated successfully by PCR-RFLP with *Mse*I, *Mun*I, *Ava*II, and *Hinc*II, with the exception of the AG-3 and AG-7 isolates from Illinois. We observed that the restriction patterns obtained for the isolates of these two AGs did not match any of the three RFLP types reported by Guillemaut et al. (2003) for AG-3, AG-7, or for the other AGs. Surprisingly, AG-7 isolates recovered from Arkansas could be resolved by PCR-RFLP, as was the AG-7 tester isolate, ST81548. Guillemaut et al. (2003) used AG-3 isolates collected from potato, tobacco, common beet, and soil to develop three RFLP patterns for this group. Similarly, the AG-7 isolates they used included those collected from soil and a single isolate recovered from soybean in Arkansas. Therefore, our inability to resolve the AG-3 isolates may be because the isolates were recovered from soybean. Also, the difficulty in identifying AG-7 isolates from Illinois may imply the presence of polymorphisms in the restriction sites of the enzymes used and further suggests that more than two RFLP types can be assigned to this AG.

For the *R. solani* isolates identified, five AGs (AG-2-2, AG-3, AG-4, AG-7, and AG-11), two subgroups within AG-2 (AG-2-1 and AG-2-2IIIB), one subgroup within AG-3 (AG-3 PT),

and two subgroups of AG-4 (AG-4 HGI and AG-4 HGIII) were identified. The diversity of AGs recovered in this study is consistent with reports from other authors (Nelson et al. 1996; Liu and Sinclair 1991; Muyolo et al. 1993; Rizvi and Yang 1996; Carling et al. 1994; Zhao et al. 2005). Combining all locations in which *R. solani* was recovered, isolates of AG-2-2IIIB (65%) represented the most frequently isolated population while AG-2-1 (1%) represented the least recovered group; however, considering each location, the number and diversity of AGs recovered varied. For example, the Illinois isolates represented five AG/subgroups (AG-2-2IIIB (48 isolates), AG-3 PT (5 isolates), AG-4 (5 isolates), AG-7 (4 isolates), AG-11 (6 isolates)), Arkansas isolates represented four AGs (AG-2-1 (1), AG-7 (5), AG-11 (2)), and the Ontario isolates represented one AG (AG-2-2IIIB (23)). The frequency of recovery for the AG-2-2IIIB isolates in Illinois and Ontario is similar to that reported in Ohio (Muyolo et al. 1993) and Ontario (Zhao et al. 2005). Muyolo et al. (1993) identified AG-2-2IIIB as the predominant AG of *R. solani* in Ohio, and this was confirmed by Dorrance et al (2003) a decade later. In Ontario, Canada, 86% of the 278 *R. solani* isolates recovered from diseased soybean plants were characterized as members of AG-2-2IIIB, while 1.4% and 12.6% belonged to AG-4 and AG-5 (Zhao et al. 2005), respectively. However, these observations are in contrast to observations from other U.S. soybean growing regions. In the Red River Valley of North Dakota and Minnesota, isolates of *R. solani* AG-2, AG-3, AG-4, and AG-5, in addition to other species of *Rhizoctonia*, were recovered from diseased soybean plants obtained from 200 fields, with AG-4 isolates being recovered more frequently (Nelson et al. 1996). Similarly, in Iowa, AG-4 was the predominant group isolated from diseased soybean seedlings (Rizvi and Yang 1996). In our study, no AG-5 isolate was recovered, and very few AG-4 isolates were recovered from Illinois. The reasons for the disparity in the predominant AGs across different soybean growing regions is uncertain but

may be attributed to differences in cropping systems (Ogoshi 1987) or soil type. The diversity of AGs recovered from Arkansas is consistent with previous reports. In the U.S., isolates of AG-7 (Rothrock et al. 1993) and AG-11 (Carling et al. 1994) were first reported on soybean seedlings in Arkansas. Also, AG-11 isolates have been associated with fields under repeated rice-soybean rotations in Arkansas (Spurlock et al. 2016) and in Texas (Jones and Carling 1999). However, this is the first report of AG-2-1 in this region and the first time AG-2-1 was isolated from soybean seedlings. AG-2-1 is known to be pathogenic on a number of host crops (Sneh et al. 1991), including peas (Hwang et al. 2007; Sharma-Poudyal et al. 2015), canola (Paulitz et al. 2006), tulip (Nakatomi and Kaneko 1971), and crucifers (Ogoshi 1987; Watanabe and Matsuda 1966), and no researchers have reported this AG as a major pathogen of soybean. The role of this AG in the ecology of *R. solani* on soybean deserves further investigation. The diversity of AGs observed in Illinois is unexpected given the less diverse cropping pattern typical of this region. With the corn-soybean rotation being the primary rotation regime in Illinois, the detection of AGs known to be pathogenic on other crops not cultivated in this region is rather surprising. Isolates of AG-3 are well established pathogens of members of the Solanaceae family (Anderson 1982; Kuninaga et al. 2000; Meyer et al. 1990; Ogoshi 1987), AG-7 are pathogens of cotton and watermelon (Baird et al. 1996; Baird and Carling 1995, 1997), and AG-11 are pathogens of lupine, cotton, and radish (Sweetingham 1989; Carling et al. 1994). The identification of these AGs on diseased soybean seedlings may imply an expansion of host range to include soybean. The recovery of only AG-2-2IIIB isolates from Ontario suggests that there has not been a change in the AGs of *R. solani* causing seedling diseases of soybean in that region. The single isolate recovered from Kansas and Michigan were identified as BR, and the reason for the low frequency of *R. solani* isolate recovery was probably due to isolation protocol or the prevalence

of climatic conditions unfavorable for their growth and detection. Isolates of BR were recovered from the different sampling locations, except Ontario. Binucleate *Rhizoctonia* or the *Ceratobasidium* spp. are *Rhizoctonia*-like fungi with binucleate cells (Sneh et al. 1991), and similar to *R. solani*, members of the genus Ceratobasidium have been classified into seven *Ceratobasidium* anastomosis groups (CAG-1 to CAG-7) (Burpee et al. 1980a), although these were later expanded to 17 AGs (AG-A to AG-Q) (Ogoshi et al. 1983). Concurrent with our observations, Nelson et al. (1996) reported the presence of BR on the roots and stems of soybean in the Red River Valley of Minnesota and North Dakota, and Ploetz et al. (1985) recovered isolates of CAG-3 from soybean and those of CAG-3 and CAG-4 from soils cultivated to rye and soybean under a reduced tillage system. The isolation of *R. zeae* from soybean seedlings was infrequent. Moreover, all *R. zeae* isolates identified in this study were recovered from Illinois, and this is the first report of this *Rhizoctonia* species on soybean seedlings in this region. *R. zeae* are multinucleate *Rhizoctonia* that are differentiated from *R. solani* by their distinctive salmon-colored mycelia in culture (Burpee and Martin 1992). Members of this species are major pathogens of corn and turfgrass species (Burpee and Martin 1992; Martin and Lucas 1984; Sumner and Bell 1982; Voorhees 1934), and the pathogenic potential on soybean is unknown. Their frequent isolation from organic debris and soils associated with symptomatic host plants (Martin and Lucas 1984b) suggests an excellent saprophytic potential. Given that corn is a major rotational crop in Illinois, *R. zeae* recovered on symptomatic soybean seedlings might have originated from the inoculum on corn residue.

From the greenhouse pathogenicity experiments, there was considerable variation in the aggressiveness among the different *R. solani* AGs and species of *Rhizoctonia*. We found that the most frequently recovered group, AG-2-2IIIB, was also the most aggressive on soybean roots

and hypocotyls. At the initial stages of our greenhouse assays, we observed that most of the AG-2-2IIIB isolates caused seed rot, preventing germination and emergence (data not shown); hence, the inoculum level was adjusted to ensure that the seeds had sufficient time to emerge before infection. This implicates this group of isolates as potential seed and root rotters. Also, AG-2-2IIIB isolates exhibited significant variability in their aggressiveness on soybean, and virulence was not correlated with isolate origin. Isolates 42304h and K_IL_S02_25b caused mild lesions on the roots, while DK_3b was moderately pathogenic. All other AG-2-2IIIB isolates produced disease symptoms comparable to 65L-2 (BF09476), an aggressive seed and root rotter. Variability in aggressiveness of AG-2-2IIIB isolates has been reported previously (Nelson et al. 1996; Dorrance et al. 2003; Muyolo et al. 1993). These observations highlight the importance of determining the aggressiveness of an isolate if the goal of the breeding program is to select for resistant soybean genotypes. The AG-4 isolates were not as aggressive as the AG-2-2IIIB isolates. Similar to what has been observed by other researchers (Fenille et al. 2003; Fenille et al. 2002; Muyolo et al. 1993), AG-4 isolates caused more severe symptoms on the hypocotyls while AG-2-2IIIB isolates caused more severe symptoms on the roots (Fig. 2.6). Although the five AG-4 isolates tested represented two homogenous groups, AG-4 HGI and AG-4 HGIII, there were no differences in aggressiveness among the five AG-4 isolates evaluated in the greenhouse. Disease symptoms produced by AG-7 and AG-11 isolates were comparable to those caused by the AG-4 isolates. This contradicts previous observations by other researchers, where these two AGs were found to be less aggressive than AG-4 on soybean seedlings (Carling et al. 1994; Rothrock et al. 1993). The long-term coexistence with soybean in the absence of other major host plants may have played a role in expanding the host range of these two AGs; however, since we did not evaluate the pathogenicity of isolates of AG-7 and AG-11 recovered outside of

Illinois, it is impossible to validate our hypothesis. Pathogenicity tests did not implicate the BR and *R. zeae* as pathogens of soybean. Binucleate *Rhizoctonia* and *R. zeae* generally are not considered pathogens of soybean, even though they have been recovered from soybean seedlings at different locations (Naito et al. 1993; Nelson et al. 1996). Isolates of certain CAGs of BR have been shown to be pathogenic on a number of host crops (Burpee et al. 1980b; Kataria and Hoffmann 1988; Sneh et al. 1991) while those of *R. zeae* mostly attack corn and turfgrass species (Burpee and Martin 1992; Martin and Lucas 1984; Sumner and Bell 1982; Voorhees 1934). Naito et al. (1993) identified isolates of AG-E from Indonesia as potential pathogens of soybean. However, the BR isolates evaluated in our greenhouse assays are members of CAG-2 (AG-A), suggesting that soybean is not a potential host to this AG. The potential of certain BR as biocontrol agents for the management of plant pathogens, including *R. solani*, has been documented (Burpee and Goulty 1984; Cardoso and Echandi 1987; Escande and Echandi 1991; Muslim et al. 2003), but experiments to validate the biocontrol potential of the recovered BR isolates in our research were not conducted. Although isolates of BR and *R. zeae* produced little to no necrotic lesions on corn roots, we observed a significant reduction in dry root weight of plants inoculated with these groups compared to the non-inoculated control. Our observations with these two species of *Rhizoctonia* suggest that root weight reduction may be due to the production of root growth inhibitory compounds.

Results of pathogenicity tests revealed that corn is a host to the AGs that are pathogenic on soybean, especially AG-2-2IIIB, AG-4, and AG-11. The AG-4 and AG-2-2IIIB isolates were highly aggressive on corn, severely rotting both the crown and seminal roots. Isolates of AG-2-2IIIB are known to cause root rot of corn in the U.S. (Ohkura et al. 2009; Sumner and Bell 1982) and in Europe (Buddemeyer et al. 2004; Ithurrart et al. 2004), and AG-4 isolates have been

reported to produce root rot symptoms that are not as severe as those produced by AG-2-2IIIB isolates. Since we tested only one isolate of AG-4, which belongs to HGI, we were unable to correlate HG-types to virulence on corn or make conclusions regarding variation in aggressiveness of HG types of AG-4 on corn. Root damage caused by AG-11 isolates was mild and mostly restricted to the mesocotyl. The identification of corn as a susceptible host to the most aggressive AGs on soybean indicates a high potential for inoculum build-up in the corn-soybean rotations in Illinois. Therefore, management practices that reduce inoculum build-up of these AGs on farmers' fields deserve consideration in this region.

Molecular characterization using the ITS sequence of the ribosomal genes has been identified as the most reliable approach for the phylogenetic studies of AGs and subgroups of different *Rhizoctonia* species (Gonzalez et al. 2001; Sharon et al. 2006). Using the ITS sequence data of the recovered isolates and those from the NCBI database, we were able to corroborate the results obtained using other characterization techniques and make inferences regarding the phylogenetic relationships among the isolates. Phylogenetic analysis allowed for the identification of certain *R. solani* isolates that remained unidentified using other AG-typing techniques. For example, the four AG-7 isolates from Illinois grouped strongly with AG-7 isolates collected from Arkansas and the AG-7 reference isolate, which also was recovered from Arkansas (AF153793). A second AG-7 cluster comprised an AG-7 tester isolate, ST81548, and an AG-7 reference isolate from Japan (AF354098) also was observed. The origin of ST81548 is unknown, but it does appear that the two AG-7 clusters observed in this study correspond to different AG-7 subgroups. Additional work involving representative isolates from different hosts and origins needs to be done to test this hypothesis. Cultural characters were too subtle to sufficiently differentiate between subgroups of AG-4, but the ITS sequence permitted their

differentiation. We identified, for the first time in the U.S., members of AG-4 HGI on soybean. In the U.S., AG-4 isolates from soybean have been characterized as HGII and HGIII, while those associated with hypocotyl rot in Brazil have been identified as HGI. We were unable to achieve successful anastomosis of the recovered BR isolates and the tester BR isolates; however, using the ITS sequence data, we identified them as AG-A, AG-F, and AG-L. Phylogenetic analysis also revealed different clades that corresponded to well-established AGs of different *Rhizoctonia* species. Based on PP values, there was a strong support (> 95%) for AG-2-1, AG-2-2IIIB, AG-3, AG-4, AG-7, and AG-11 clusters. The AG-2-1 and AG-2-2IIIB isolates did not cluster together on the phylogenetic tree despite being members of the same AG. This observation is not surprising given that polyphyly among members of this AG has been reported by several authors (Budge et al. 2009; González et al. 2006; Kuninaga et al. 1997; Ohkura et al. 2009; Salazar et al. 2000; Salazar et al. 1999; Sharon et al. 2008; Stodart et al. 2007). Although there was a strong support (100%) for the separation of the BR and *R. solani* isolates, certain BR isolates clustered with isolates of *R. solani*. This suggests that certain BR are more closely related to some *R. solani* isolates than they are to other members of *Ceratobasidium* spp. Two AG-F isolates (X12RS42 and KARS02_1_19) clustered in the same clade as the AG-4 and AG-7 isolates, although this grouping was weakly supported (PP = 65%). Another AG-F isolate (9SDS) clustered strongly (99%) with *R. solani* AG-6. Similar observations indicating genetic relatedness between BR and some *R. solani* isolates have been reported by several authors. For example, Gonzalez et al. (2001) found that certain BR belonging to the *Ceratobasidium* anastomosis groups 4 and 6 (CAG-4 and CAG-6) clustered closely with *R. solani* AG-4 isolates. Similarly, cluster analysis of rDNA-ITS region sequences of *Rhizoctonia* isolates recovered from strawberries in Israel grouped *Ceratobasidium* sp. AG-F isolates more closely with *R. solani*

AG-4 isolates than with other *Ceratobasidium* spp. (Sharon et al. 2007). Also, the genetic relatedness between BR AG-F and *R. solani* AG-6 has been suggested based on hyphal anastomosis reactions (Yokoyama and Ogoshi 1986). These observations all together indicate that BR represents a polyphyletic group and that further studies are required to clarify their phylogenetic relationship with *R. solani*.

In summary, our work provides evidence for the association of a diverse group of *Rhizoctonia* species with soybean, with AG-2-2IIIB as the most predominant and aggressive group. Two groups (AG-2-1 and AG-4 HGI) previously unidentified as part of the soybean seedling disease complex and two divergent groups of AG-7 were identified in this study. The susceptibility of corn to AG-2-2IIIB, and other AGs pathogenic on corn, highlights the potential risks of the corn-soybean rotation, especially in areas where such AGs are prevalent.

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## Tables

**Table 2.1.** Origin, year of isolation, and identification method used for the isolates of *Rhizoctonia* collected from soybean in Arkansas, Illinois, Kansas, Michigan, Minnesota, and Ontario.

| Isolate ID | Year isolated | County/State | *R. solani* AG/ Rhizoctonia species | Identification method |
|---|---|---|---|---|
| 12RS42 | 2012 | Champaign, IL | *Ceratobasidium* sp. | ITS, CNN |
| 211 | 2012 | Champaign, IL | *R. zeae* | M |
| C_far 500_10a | 2013 | Champaign, IL | AG-3 | ITS, RFLP-PCR |
| C_far 500_3 | 2013 | Champaign, IL | AG-3 | ITS, RFLP-PCR |
| C_far 500_6 | 2013 | Champaign, IL | AG-3 | ITS, RFLP-PCR |
| C_far 500_10 | 2013 | Champaign, IL | AG-3 | ITS, RFLP-PCR |
| IL 2014a | 2014 | Champaign, IL | AG-2-2IIIB | RFLP-PCR |
| 12RS39 | 2012 | DeKalb, IL | *R. zeae* | M |
| DK_10 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_11 | 2013 | DeKalb, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| DK_13 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| Dk_14 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_15a | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_15b | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_16 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_19 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_3a | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_3b | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_4a | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_4b | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_6a | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_6b | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| Dk_7 | 2013 | DeKalb, IL | AG-2-2IIIB | RFLP-PCR |
| DK_8 | 2013 | DeKalb, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| K_2_1a | 2013 | Gallatin, IL | *Ceratobasidium* sp. | ITS,CNN |
| W_2_2_15 | 2013 | Gallatin, IL | *Ceratobasidium* sp. | CNN |
| W2_2_20 | 2013 | Gallatin, IL | *Ceratobasidium* sp. | CNN |

[a]CNN = cellular nuclear number; M = morphological identification on culture media; ITS = internal transcribed spacer region of the ribosomal RNA genes.

**Table 2.1 (cont.)**

| Isolate ID | Year isolated | County/State | *R. solani* AG/ Rhizoctonia species | Identification method |
|---|---|---|---|---|
| 12RS52 | 2012 | Jackson, IL | AG-11 | RFLP-PCR |
| 12RS40 | 2012 | Jackson, IL | *R. zeae* | M |
| 248_1a KH | 2012 | Jackson, IL | *R. zeae* | M |
| 248_3b KH | 2012 | Jackson, IL | AG-3 | ITS |
| 248_2KH | 2012 | Jackson, IL | *R. zeae* | M |
| EV_19 | 2013 | Jackson, IL | AG-7 | ITS |
| EV_3 | 2013 | Jackson, IL | AG-4 HGI | ITS, RFLP-PCR |
| EV_6 | 2013 | Jackson, IL | AG-4 HGI | ITS, RFLP-PCR |
| EV_7 | 2013 | Jackson, IL | AG-7 | ITS |
| 12RS36 | 2012 | McLean, IL | *R. zeae* | M |
| 12RS48 | 2012 | Peoria, IL | *R. zeae* | M |
| S_P_18a | 2013 | Pike, IL | *Ceratobasidium* sp. | ITS, CNN |
| S_P_18b | 2013 | Pike, IL | *Ceratobasidium* sp. | ITS, CNN |
| S_P_18c | 2013 | Pike, IL | *Ceratobasidium* sp. | ITS, CNN |
| S_P_19a | 2013 | Pike, IL | AG-11 | ITS, RFLP-PCR |
| S_P_19b | 2013 | Pike, IL | AG-11 | ITS, RFLP-PCR |
| S_P_2 | 2013 | Pike, IL | *Ceratobasidium* sp. | ITS, CNN |
| K_1_24c | 2013 | Pope, IL | *Ceratobasidium* sp. | ITS, CNN |
| W2_1_12 | 2013 | Pope, IL | *R. zeae* | M, ITS |
| W2_2_12 | 2013 | Pope, IL | *R. zeae* | M, ITS |
| Maxwell 1_1_ KH | 2012 | Sangamon, IL | *R. zeae* | M, ITS |
| NBK_IL_3 | 2013 | Sangamon, IL | *Ceratobasidium* sp. | CNN |
| NBK_IL_5 | 2013 | Sangamon, IL | *Ceratobasidium* sp. | CNN |
| K_4_18b | 2013 | St. Clair, IL | AG-11 | ITS, RFLP-PCR |
| BVT_11 | 2013 | St. Clair, IL | AG-4 HGI | RFLP-PCR |
| BVT_16 | 2013 | St. Clair, IL | AG-7 | ITS |
| BVT_18 | 2013 | St. Clair, IL | AG-11 | ITS, RFLP-PCR |
| BVT_20 | 2013 | St. Clair, IL | AG-7 | ITS |

[a]CNN = cellular nuclear number; M = morphological identification on culture media; ITS = internal transcribed spacer region of the ribosomal RNA genes.

**Table 2.1 (cont.)**

| Isolate ID | Year isolated | County/State | *R. solani* AG/ Rhizoctonia species | Identification method |
|---|---|---|---|---|
| BVT_28 | 2013 | St. Clair, IL | AG-4 HGIII | ITS, RFLP-PCR |
| BVT_3 | 2013 | St. Clair, IL | AG-4 HGIII | ITS, RFLP-PCR |
| 12SDSa | 2013 | Unknown, IL | AG-11 | ITS, RFLP-PCR |
| 12SDSb | 2013 | Unknown, IL | *Ceratobasidium* sp. | ITS, CNN |
| 2SDS | 2013 | Unknown, IL | *Ceratobasidium* sp. | CNN |
| 4SDS | 2013 | Unknown, IL | *Ceratobasidium* sp. | ITS, CNN |
| 9SDS | 2013 | Unknown, IL | Unknown | ITS, CNN |
| 42210_b | 2013 | Unknown, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| 42210_c | 2013 | Unknown, IL | AG-2-2IIIB | RFLP-PCR |
| 42304_b | 2013 | Unknown, IL | AG-2-2IIIB | RFLP-PCR |
| 42304_g | 2013 | Unknown, IL | AG-2-2IIIB | RFLP-PCR |
| K_IL_SO2_3_25b | 2013 | Warren, IL | AG-2-2IIIB | RFLP-PCR |
| m_20_h | 2012 | Whiteside, IL | *Ceratobasidium* sp. | CNN |
| m_20_k | 2012 | Whiteside, IL | *Ceratobasidium* sp. | CNN |
| m_24_b | 2012 | Whiteside, IL | *Ceratobasidium* sp. | ITS, CNN |
| m_24_c | 2012 | Whiteside, IL | *Ceratobasidium* sp. | ITS, CNN |
| ER_15 | 2013 | Whiteside, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| ER_19a | 2013 | Whiteside, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| ER_19b | 2013 | Whiteside, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| ER_2 | 2013 | Whiteside, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| ER_4 | 2013 | Whiteside, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| 12RS41 | 2012 | Williamson, IL | AG-2-2IIIB | ITS, RFLP-PCR |
| K_ARSO2_1_11 | 2013 | Arkansas | AG-11 | ITS, RFLP-PCR |
| K_ARSO2_1_19 | 2013 | Arkansas | *Ceratobasidium* sp. | ITS, CNN |
| K_ARSO2_1_20 | 2013 | Arkansas | AG-11 | ITS, RFLP-PCR |
| K_ARSO2_1_6 | 2013 | Arkansas | AG-7 | ITS, RFLP-PCR |
| K_ARSO2_1_8 | 2013 | Arkansas | AG-7 | ITS, RFLP-PCR |
| K_ARSO2_1_7 | 2013 | Arkansas | AG-7 | RFLP-PCR |

[a]CNN = cellular nuclear number; M = morphological identification on culture media; ITS = internal transcribed spacer region of the ribosomal DNA.

**Table 2.1 (cont.)**

| Isolate ID | Year isolated | County/State | *R. solani* AG/ Rhizoctonia species | Identification method |
|---|---|---|---|---|
| K_ARSO2_5_20 | 2013 | Arkansas | *Ceratobasidium* sp. | ITS, CNN |
| K_ARSO2_2_5 | 2013 | Arkansas | AG-7 | ITS, RFLP-PCR |
| K_ARSO2_1_9 | 2013 | Arkansas | AG-7 | ITS, RFLP-PCR |
| K_ARSO2_5_1 | 2013 | Arkansas | AG-2-1 | ITS, RFLP-PCR |
| W_KSSO2_2_13 | 2013 | Kansas | *Ceratobasidium* sp. | CNN |
| W_MISO2_6_19 | 2013 | Michigan | *Ceratobasidium* sp. | ITS, CNN |
| MNSO1_3_14 | 2012 | Minnesota | *Ceratobasidium* sp. | ITS, CNN |
| ONSO2_17 | 2012 | Ontario | AG-2-2IIIB | RFLP-PCR |
| ONSO2_16 | 2012 | Ontario | AG-2-2IIIB | RFLP-PCR |
| ONSO2_14 | 2012 | Ontario | AG-2-2IIIB | RFLP-PCR |
| ONSO2_13 | 2012 | Ontario | AG-2-2IIIB | RFLP-PCR |
| ONSO2_15 | 2012 | Ontario | AG-2-2IIIB | RFLP-PCR |
| ONSO2_18 | 2012 | Ontario | AG-2-2IIIB | ITS, RFLP-PCR |
| WONS 13_8_4 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_2 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_6 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_4 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_5 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_6 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_3 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_7 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_8_8 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_8 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_3 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_7 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| WONS 13_8_1 | 2013 | Ontario | AG-2-2IIIB | ITS, RFLP-PCR |
| WONS 13_8_5 | 2013 | Ontario | AG-2-2IIIB | ITS, RFLP-PCR |
| PDONS 13_8_1 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |
| PDONS 13_12_3 | 2013 | Ontario | AG-2-2IIIB | ITS, RFLP-PCR |
| WONS 13_8_4 | 2013 | Ontario | AG-2-2IIIB | RFLP-PCR |

[a]CNN = cellular nuclear number; M = morphological identification on culture media; ITS = internal transcribed spacer region of the ribosomal DNA.

**Table 2.2.** Tester isolates used for anastomosis group-typing of unknown *Rhizoctonia* isolates recovered in this study

| Tester isolates' ID | *R. solani* AG/ *Rhizoctonia* species | Source[a] |
|---|---|---|
| Rh051307 | AG-2-1 | Tim Paulitz, WSU |
| Rh051324 | AG-2-1 | Tim Paulitz, WSU |
| Rh051339 | AG4 HGII | Tim Paulitz, WSU |
| Rh051320 | AG-8 | Tim Paulitz, WSU |
| Rh061303 | AG-8 | Tim Paulitz, WSU |
| Rh0911028 | AG-10 | Tim Paulitz, WSU |
| Rh0911029 | AG-5[b] | Tim Paulitz, WSU |
| Rh051364 | *Ceratobasidium* sp; AG-A | Tim Paulitz, WSU |
| Rh1212154 | *Ceratobasidium* sp; AG-A | Tim Paulitz, WSU |
| R88-40B | *R. oryzae* | Craig Rothrock, UA |
| R63-42A | *R. zeae* | Craig Rothrock, UA |
| AG1.1A | AG-1-1A | Craig Rothrock, UA |
| AG1.1C | AG-1-1C | Craig Rothrock, UA |
| HPIN22A | AG-11 | Craig Rothrock, UA |
| PT35-25A | AG-11 | Craig Rothrock, UA |
| AG9 | AG-9 | Craig Rothrock, UA |
| V50.34 | AG-2-2 | Craig Rothrock, UA |
| AG6 | AG-6 | Craig Rothrock, UA |
| AG8 Tester | AG-8 | Craig Rothrock, UA |
| ST81548 | AG-7 | Craig Rothrock, UA |
| AG4 Pathogenic tester | AG-4 | Craig Rothrock, UA |
| USA AG3 | AG-3 | Berlin Nelson, NDSU |
| USA AG5 | AG-5 | Berlin Nelson, NDSU |
| AG-B1 | AG-BI | Berlin Nelson, NDSU |
| 65L-2 (BF09476)[c] | AG-2-2IIIB | Sinclair, UIUC |

[a] WSU = Washington State University; UA = University of Arkansas; NDSU = North Dakota State University; UIUC = University of Illinois at Urbana-Champaign

[b] Rh0911029 was originally characterized as AG-10, but this was identified as AG-5 using PCR-RFLP and phylogenetic analysis of the ITS region

[c] 65L-2 was originally collected by Liu and Sinclair (1991)

**Table 2.3.** Reference isolates of *Rhizoctonia* and other fungi retrieved from GeneBank for the characterization of the isolates recovered from Arkansas, Illinois, Michigan, Minnesota, and Ontario.

| GeneBank accession number | Fungal species |
|---|---|
| AY684917 | *Athelia rolfsii* |
| AB213594 | *Waitea circinata* var. *zeae* |
| KC193238 | *Ceratobasidium* sp. (AG-F) |
| DQ102433 | *Ceratobasidium* sp. (AG-F) |
| AB286933 | *Ceratobasidium* sp. (AG-L) |
| AF354092 | *Ceratobasidium* sp. (AG-A) |
| AB122142 | *R. solani* AG1-1C |
| AY154317 | *R. solani* AG-2-1 |
| AF354116 | *R. solani* AG-2-2IIIB |
| GU811670 | *R. solani* AG-2-2IIIB |
| GQ885147 | *R. solani* AG-3 |
| AY154659 | *R. solani* AG-4 HGIII |
| HQ629873 | *R. solani* AG-4 HGII |
| AB000007 | *R. solani* AG-4 HGI |
| AF354113 | *R. solani* AG-5 |
| AF354102 | *R. solani* AG-6 |
| AF153793 | *R. solani* AG-7 |
| AF354098 | *R. solani* AG-7 |
| FJ435117 | *R. solani* AG-8 |
| FJ435097 | *R. solani* AG-9 |
| AF153800 | *R. solani* AG-10 |
| AF354114 | *R. solani* AG-11 |
| AF153807 | *R. solani* AG-12 |
| AF354110 | *R. solani* AG-BI |

**Table 2.4**. Median, mean rank, and estimated relative effect for the severity of hypocotyl and root rot symptoms caused by isolates of different *Rhizoctonia* spp. on soybean

| Isolate | Anastomosis group | Median disease rating[a] | Estimated relative effect $(\hat{p}_{ij})$[b] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[c] | |
|---|---|---|---|---|---|---|
| | | | | | lower limit | upper limit |
| Control | - | 0 | 0.04 | 14.4 | 0.03 | 0.07 |
| 12SDSa | AG11 | 1 | 0.30 | 99 | 0.28 | 0.32 |
| K_4_18b | AG11 | 1.75 | 0.45 | 148.5 | 0.33 | 0.58 |
| SP_19a | AG11 | 1 | 0.25 | 84.4 | 0.18 | 0.35 |
| 211_1a | AG-2-2IIIB | 4 | 0.71 | 229.3 | 0.62 | 0.79 |
| 42210b | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| 42304h | AG-2-2IIIB | 0.75 | 0.18 | 58.2 | 0.09 | 0.31 |
| BF09476 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| DK_11 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| DK_13 | AG-2-2IIIB | 4.5 | 0.75 | 241.3 | 0.65 | 0.82 |
| DK_14 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| DK_15a | AG-2-2IIIB | 4.75 | 0.72 | 233.6 | 0.6 | 0.82 |
| DK_15b | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| DK_16 | AG-2-2IIIB | 5 | 0.82 | 262.6 | 0.75 | 0.87 |
| DK_19 | AG-2-2IIIB | 3.25 | 0.61 | 199.3 | 0.47 | 0.74 |
| DK_3a | AG-2-2IIIB | 5 | 0.82 | 263.9 | 0.76 | 0.87 |

[a] Median disease rating was on a scale of 0 to 5

[b]Relative effects $(\hat{p}_{ij})$ were determined as described by Shah and Madden (2004).

[c]Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

**Table 2.4 (cont.)**

| Isolate | Anastomosis group | Median disease rating[a] | Estimated relative effect $(\hat{p}_{ij})$[b] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[c] | |
|---|---|---|---|---|---|---|
| | | | | | lower limit | upper limit |
| DK_3b | AG-2-2IIIB | 2.5 | 0.54 | 178.4 | 0.44 | 0.65 |
| DK_4a | AG-2-2IIIB | 4.5 | 0.75 | 242.5 | 0.66 | 0.82 |
| DK_6 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| DK_8 | AG-2-2IIIB | 5 | 0.82 | 262.6 | 0.75 | 0.87 |
| ER_15 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| ER_19a | AG-2-2IIIB | 4 | 0.67 | 216.1 | 0.48 | 0.81 |
| ER_19b | AG-2-2IIIB | 5 | 0.82 | 263.9 | 0.76 | 0.87 |
| ER_2 | AG-2-2IIIB | 5 | 0.85 | 272 | 0.83 | 0.86 |
| ER_4 | AG-2-2IIIB | 5 | 0.79 | 253.2 | 0.7 | 0.85 |
| K_IL_S02_25b | AG-2-2IIIB | 1 | 0.25 | 84.4 | 0.18 | 0.35 |
| K_IL_S02_3_25c | AG-2-2IIIB | 5 | 0.82 | 263.9 | 0.76 | 0.87 |
| 248_3bKH | AG-3 | 1 | 0.3 | 99 | 0.28 | 0.32 |
| C_far_500_10a | AG-3 | 1 | 0.22 | 72.8 | 0.14 | 0.33 |
| C_far_500_3 | AG-3 | 1 | 0.26 | 87.3 | 0.2 | 0.34 |
| C_far_500_6 | AG-3 | 1 | 0.3 | 99 | 0.28 | 0.32 |
| USA_AG3 | AG-3 | 1 | 0.3 | 99 | 0.28 | 0.32 |

[a] Median disease rating was on a scale of 0 to 5

[b] Relative effects $(\hat{p}_{ij})$ were determined as described by Shah and Madden (2004).

[c] Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

**Table 2.4 (cont.)**

| Isolate | Anastomosis group | Median disease rating[a] | Estimated relative effect $(\hat{p}_{ij})$[b] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[c] | |
|---|---|---|---|---|---|---|
| | | | | | lower limit | upper limit |
| BV_T11 | AG-4 HGI | 1 | 0.34 | 112.7 | 0.27 | 0.43 |
| EV_3 | AG-4 HGI | 1.75 | 0.49 | 162.5 | 0.42 | 0.57 |
| EV_6 | AG-4 HGI | 1.5 | 0.49 | 158.4 | 0.32 | 0.66 |
| BVT_28 | AG-4 HGIII | 1.25 | 0.37 | 121.1 | 0.25 | 0.51 |
| BVT_3 | AG-4 HGIII | 1 | 0.34 | 112.7 | 0.27 | 0.43 |
| BVT_16 | AG-7 | 2 | 0.52 | 170.6 | 0.43 | 0.6 |
| BVT_20 | AG-7 | 1 | 0.38 | 126.3 | 0.29 | 0.49 |
| EV_19 | AG-7 | 2 | 0.5 | 164.9 | 0.42 | 0.58 |
| EV_7 | AG-7 | 1 | 0.34 | 112.7 | 0.27 | 0.43 |
| K2_1a | AG-A | 0.25 | 0.13 | 43.6 | 0.06 | 0.27 |
| K_1_24c | AG-A | 1 | 0.25 | 84.4 | 0.18 | 0.35 |
| M_24_C | AG-A | 1 | 0.26 | 87.3 | 0.2 | 0.34 |
| SP_18c | AG-A | 1 | 0.3 | 98.6 | 0.21 | 0.41 |
| W_2_2_15 | AG-A | 0.5 | 0.17 | 55.3 | 0.08 | 0.31 |
| 12RS40 | *R. zeae* | 0.5 | 0.14 | 46.5 | 0.07 | 0.27 |
| 248_2KH | *R. zeae* | 1 | 0.22 | 72.8 | 0.14 | 0.33 |
| W2_1_12 | *R. zeae* | 1 | 0.25 | 84.4 | 0.18 | 0.35 |

[a] Median disease rating was on a scale of 0 to 5

[b] Relative effects $(\hat{p}_{ij})$ were determined as described by Shah and Madden (2004).

[c] Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

**Table 2.5.** Median, mean rank, and estimated relative effect for the severity of hypocotyl and root rot symptoms caused by isolates of different *Rhizoctonia* spp. on corn

| Isolate | Anastomosis group | Median disease rating[a] | Estimated relative effect $(\hat{p}_{ij})$[b] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[c] | |
|---|---|---|---|---|---|---|
| | | | | | lower limit | upper limit |
| Control | Control | 0 | 0.25 | 26.0 | 0.18 | 0.34 |
| 12SDSa | AG-11 | 2 | 0.68 | 69.5 | 0.64 | 0.71 |
| K_4_18b | AG-11 | 1 | 0.50 | 51.6 | 0.39 | 0.61 |
| SP_19a | AG-11 | 2.5 | 0.73 | 75.2 | 0.66 | 0.79 |
| BF09476 | AG-2-2IIIB | 4.5 | 0.92 | 94.3 | 0.89 | 0.94 |
| DK_3a | AG-2-2IIIB | 3.5 | 0.82 | 84.0 | 0.79 | 0.85 |
| ER_2 | AG-2-2IIIB | 3 | 0.81 | 82.8 | 0.77 | 0.84 |
| Cfar_500 | AG-3 | 0 | 0.25 | 26.0 | 0.18 | 0.34 |
| USA_AG3 | AG-3 | 0.25 | 0.38 | 39.2 | 0.25 | 0.53 |
| X248_3bK | AG-3 | 0 | 0.25 | 26.0 | 0.18 | 0.34 |
| EV_6 | AG-4 HGI | 5 | 0.96 | 98.0 | 0.94 | 0.96 |
| BVT_20 | AG-7 | 1 | 0.54 | 55.3 | 0.41 | 0.66 |
| EV_19 | AG-7 | 0 | 0.25 | 26.0 | 0.18 | 0.34 |
| K2_1a | AG-A | 0 | 0.27 | 28.3 | 0.18 | 0.40 |
| W_2_2_15 | AG-A | 0 | 0.26 | 26.7 | 0.18 | 0.35 |
| X12RS40 | *R.zeae* | 0 | 0.25 | 26.0 | 0.18 | 0.34 |
| X248_2KH | *R.zeae* | 0.5 | 0.39 | 40.8 | 0.26 | 0.55 |

[a] Median disease rating was on a scale of 0 to 5

[b] Relative effects ($\hat{p}_{ij}$) were determined as described by Shah and Madden (2004).

[c] Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

**Table 2.6**. Variation in cellular nuclear number among anastomosis groups of *Rhizoctonia solani* identified in this study.

| Group | Cellular nuclear number | |
|---|---|---|
| | **Range** | **Mode** |
| AG-2-2IIIB | 4 to 12 | 8 |
| AG-3 | 5 to 10 | 7 |
| AG-4 | 4 to 6 | 5 |
| AG-7 | 4 to 6 | 10 |
| AG-11 | 5 to 10 | 8 |
| Binucleate *Rhizoctonia* | 2 | 2 |
| *R. zeae* | 4 to 7 | 5 |

**Table 2.7.** Estimated relative effects and mean rank for the severity of hypocotyl and root rot symptoms on soybean by isolates of different *Rhizoctonia* spp. grouped by AG/subgroup/species

| Group | Estimated relative effect $(\hat{p}_{ij})$[a] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[b] | |
|---|---|---|---|---|
| | | | lower | upper |
| Control | 0.04 e | 14.4 | 0.03 | 0.07 |
| *R. zeae* | 0.21 d | 67.9 | 0.16 | 0.26 |
| Binucleate *Rhizoctonia* | 0.23 d | 76.1 | 0.20 | 0.27 |
| AG-3 | 0.28 cd | 91.4 | 0.25 | 0.31 |
| AG-11 | 0.34 bc | 110.6 | 0.28 | 0.40 |
| AG-4 | 0.41 b | 135.8 | 0.37 | 0.46 |
| AG-7 | 0.44 b | 143.6 | 0.39 | 0.49 |
| AG-2-2IIIB | 0.73 a | 236.0 | 0.71 | 0.75 |

[a] Isolates were initially grouped by AG or species before determining relative effects $(\hat{p}_{ij})$ as described by Shah and Madden (2004).

[b]Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

**Table 2.8**. Estimated relative effects and mean rank for the severity of hypocotyl and root rot symptoms on corn by isolates of different *Rhizoctonia* spp. grouped by AG/subgroup/species

| Group | Estimated relative effect $(\hat{p}_{ij})$[a] | Mean rank | 95% Confidence interval for $(\hat{p}_{ij})$[b] | |
|---|---|---|---|---|
| | | | lower | upper |
| Control | 0.25 d | 26.0 | 0.21 | 0.27 |
| Binucleate *Rhizoctonia* | 0.28 d | 27.5 | 0.23 | 0.35 |
| AG-3 | 0.29 d | 30.4 | 0.24 | 0.34 |
| *R. zeae* | 0.32 d | 33.4 | 0.25 | 0.40 |
| AG-7 | 0.39 d | 40.7 | 0.30 | 0.49 |
| AG-11 | 0.64 c | 65.4 | 0.58 | 0.68 |
| AG-2-2IIIB | 0.85 b | 87.0 | 0.83 | 0.86 |
| AG-4 | 0.96 a | 98.0 | 0.93 | 0.97 |

[a] Isolates were initially grouped by AG or species before determining relative effects $(\hat{p}_{ij})$ as described by Shah and Madden (2004).

[b]Confidence interval for the $\hat{p}_{ij}$ of each isolate was calculated using the LD_CI macro written by Brunner et al. (2002)

## Figures

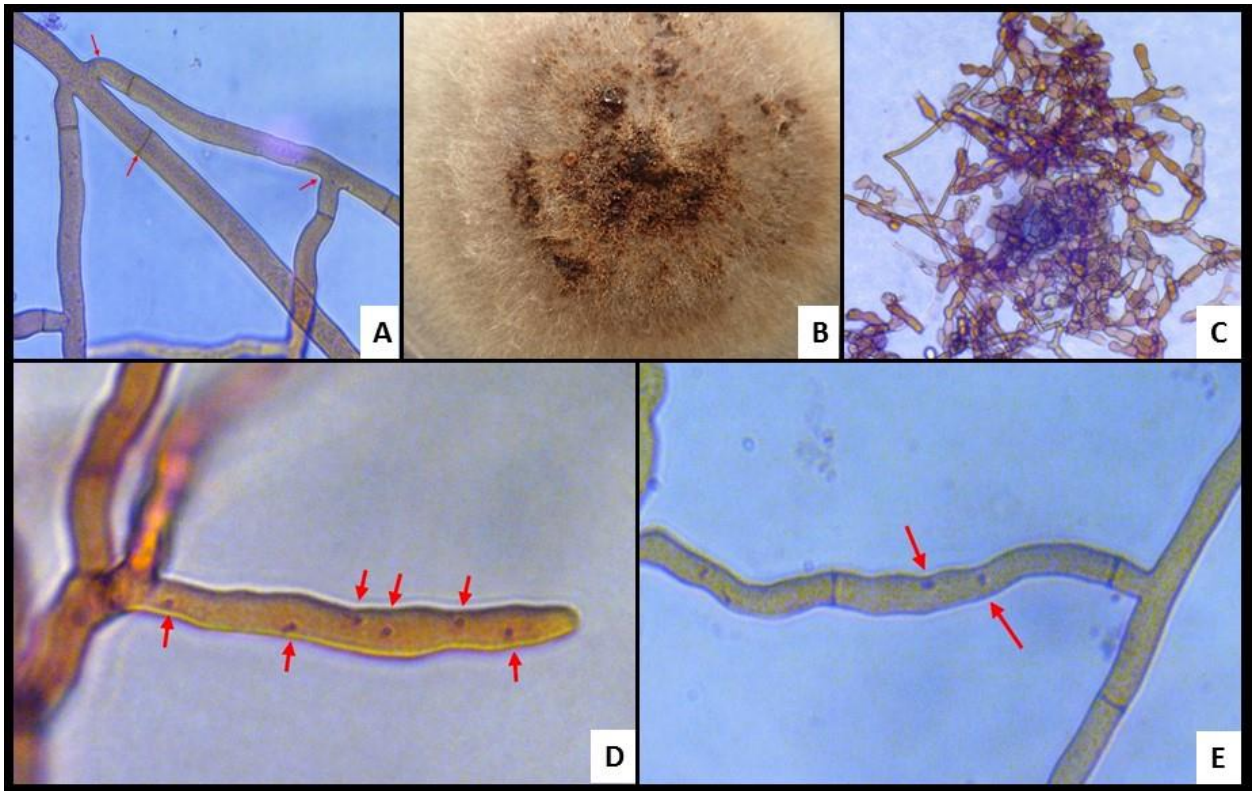


**Fig. 2.1**. Microscopic and macroscopic characteristics of *Rhizoctoni solani.* (A) Right angled branching of septate hyphae. (B) Dark brown sclerotia of a growing colony on potato dextrose agar. (C) Monilioid cells of sclerotia. (D) Multinucleate *Rhizoctonia*. (E) Binucelate *Rhizoctonia*.

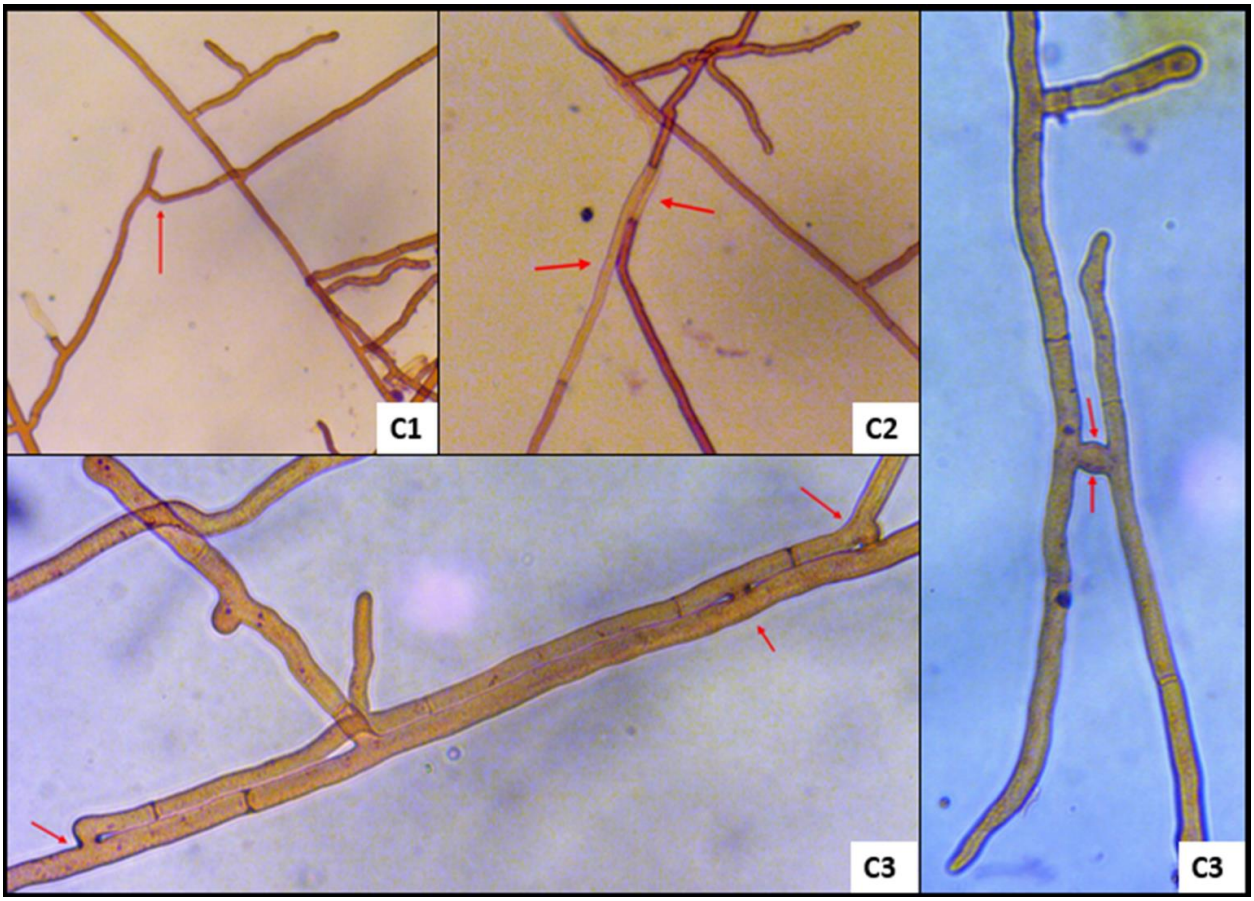


**Fig. 2.2.** Hyphal anastomosis reaction types for anastomosis group designation of *Rhizoctonia solani* isolates. (C1) Cell wall contact only. (C2) Cell wall fusion and death of anastomosing cells (killing reaction) (C3) Perfect fusion of cell walls and cell membranes, and an absence of the killing reaction.

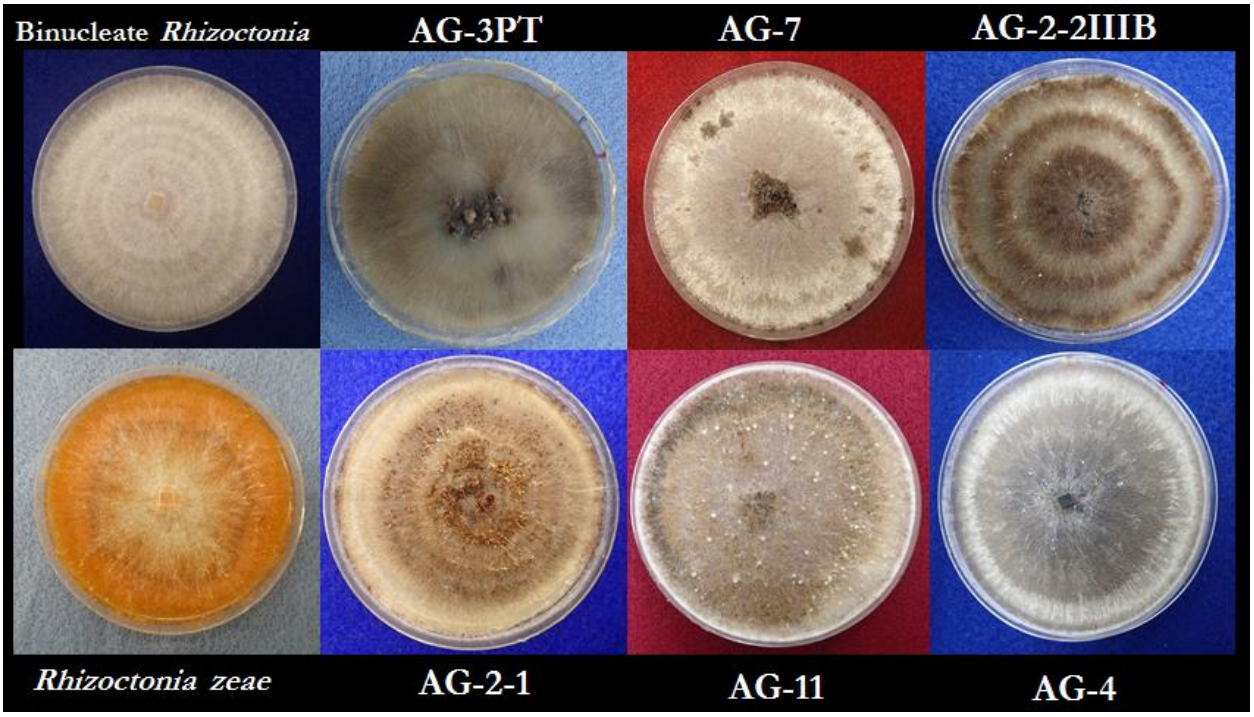


**Fig. 2.3.** Colony morphology of the binucleate *Rhizoctonia*, *Rhizoctonia zeae*, and some select anastomosis groups (AG) of *Rhizoctonia solani*

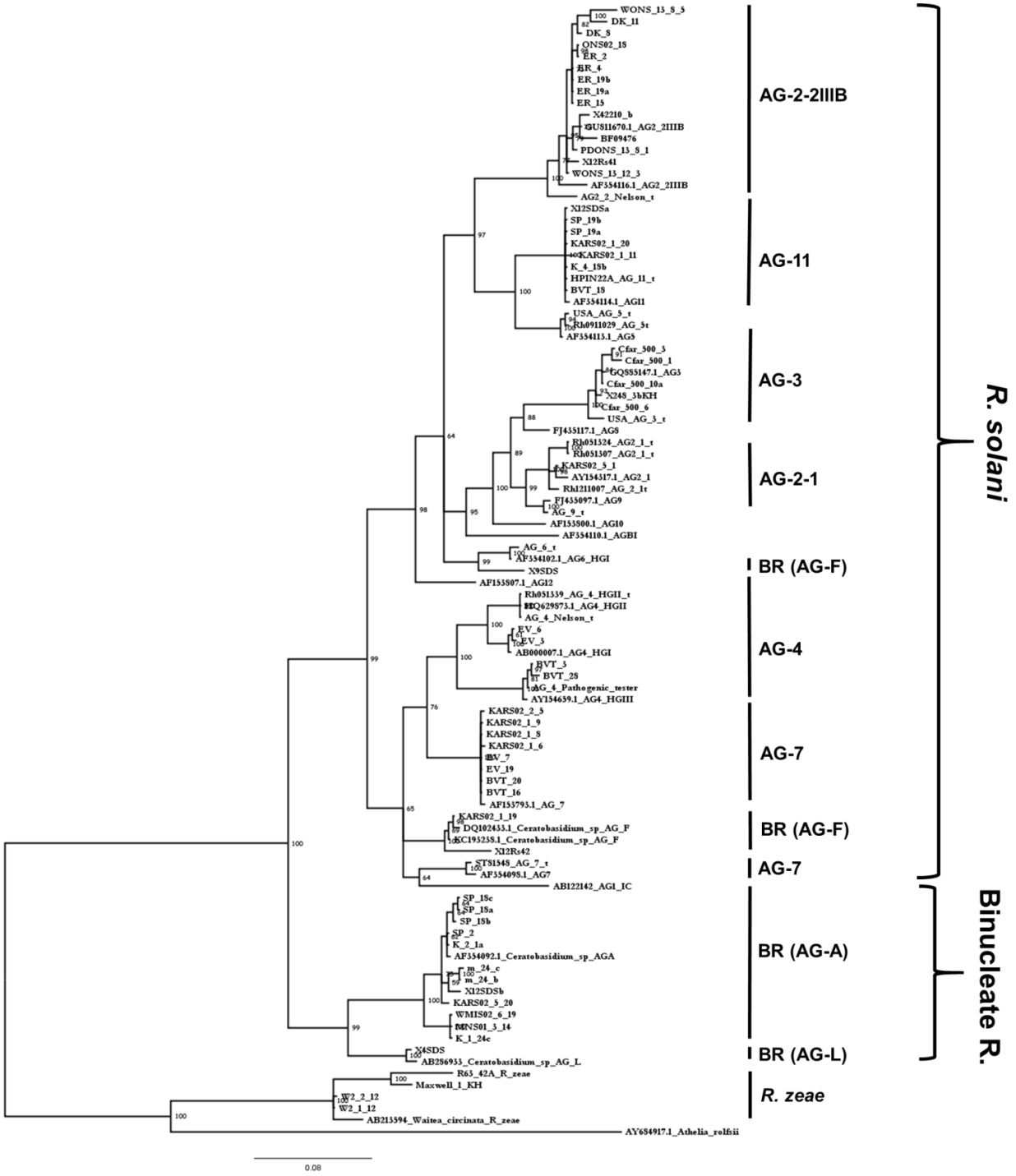


**Fig. 2.4.** Consensus tree generated from the Bayesian analysis of the rDNA ITS1-5.8S-ITS2 region of selected *Rhizoctonia* isolates from AR, IL, MI, MN, and Ontario. Reference isolates from the NCBI database are indicated with accession numbers, followed by AG/subgroup/species designation. Tester isolates are indicated by isolate name, followed by AG/subgroup and a "t". Numbers above branches represent Bayesian posterior probability values in percentage.

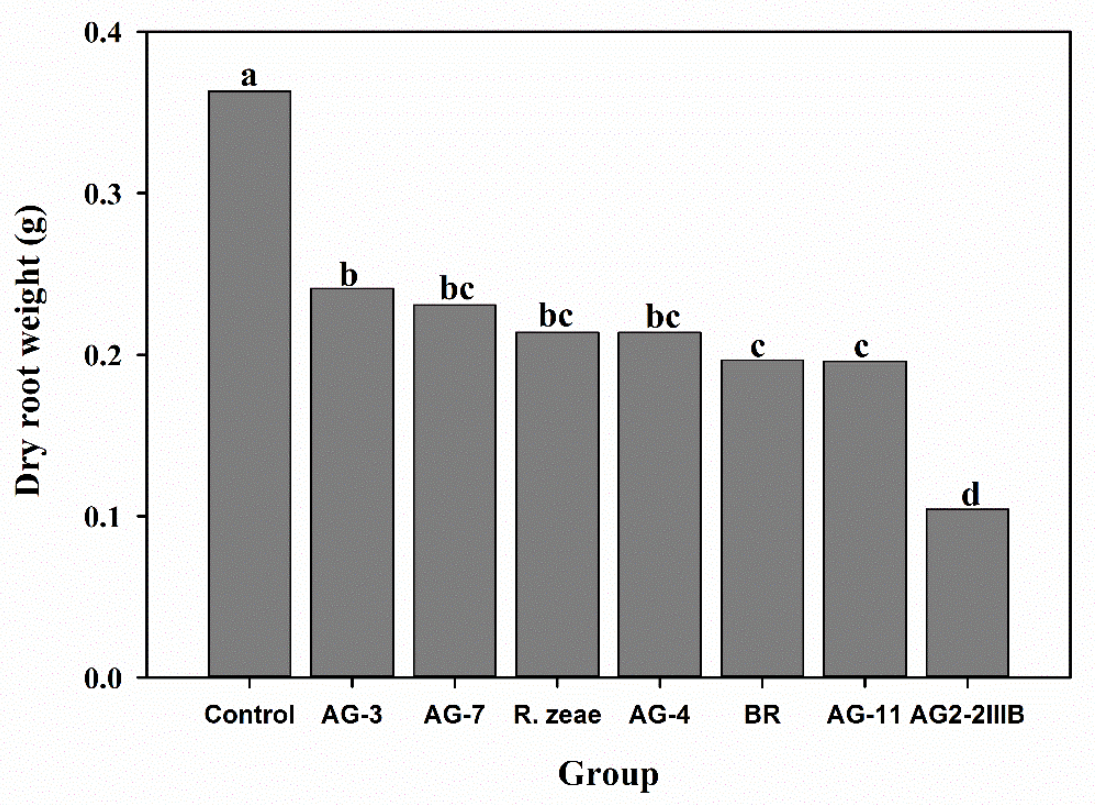


**Fig. 2.5.** Dry root weight in grams of soybean plants inoculated with different anastomosis group (AG), subgroup, and species of *Rhizoctonia* in the greenhouse. Means with the same letter are not significantly different using Fisher's protected least significant difference (LSD, $\alpha = 0.05$)


**Fig. 2.6**. Hypocotyl and root rot symptoms caused by *Rhizoctonia solani* AG-2-2IIIB and AG-4 on soybean cv. Williams 82.

# CHAPTER 3: GENETIC STRUCTURE OF *RHIZOCTONIA SOLANI* AG-2-2IIIB FROM SOYBEAN IN ILLINOIS, OHIO, AND ONTARIO


## Abstract

*Rhizoctonia solani* AG-2-2IIIB is an important seedling pathogen of soybean in North America and in other soybean-growing regions around the world. To date, there is no information regarding the population genetics of field populations of *R. solani* associated with soybean. More specifically, information regarding genetic diversity, the mode of reproduction, and the evolutionary factors that shape different *R. solani* populations separated in time and space are lacking. We exploited genotyping-by-sequencing as a tool to assess the genetic structure of *R. solani* AG2-2IIIB populations from Illinois (35), Ohio (8), and Ontario (17) and to answer the question about the reproductive mode of this subgroup. Our results revealed differences in genotypic diversity among the three populations, with the Ontario population having the greatest diversity. An overrepresentation of multilocus genotypes (MLGs) and a rejection of the null hypothesis of random mating in all three populations suggested clonality within each population. However, phylogenetic analysis revealed long terminal multifurcating branches for most of the members of the Ontario population, suggesting a mixed reproductive mode for this population. Analysis of molecular variance revealed low levels of population differentiation, and the sharing of similar MLGs among populations highlights the role of genotype flow as an evolutionary force shaping the population structure of this subgroup.


## Introduction

The soilborne necrotrophic fungus, *Rhizoctonia solani*, is a species complex comprising genetically diverse group of pathogens that have been associated with diseases of economically important crops world-wide (Sneh et al. 1991). To date, 14 genetically isolated groups, referred

to as anastomosis groups (AG), have been differentiated, many of which have been further divided into subgroups based on various phenotypic, biochemical, and molecular markers (Sneh et al. 1991; Carling et al. 1999, 2002a, b). *R. solani* anastomosis group 2, subgroup IIIB (AG-2-2IIIB) is a major seedling pathogen of soybean in North America (Liu and Sinclair 1991; Dorrance et al. 2003; Muyolo et al. 1993; Nelson et al. 1996; Zhao et al. 2005) and in other parts of the world where soybean is grown (Fenille et al. 2002). Disease symptoms, which include seed decay and pre-emergence damping off, are favored by the cool and wet conditions that occur right after planting and are often prevalent on fields with previous disease history. On emerged plants, hypocotyl rot, with characteristic sunken reddish brown lesions, is commonly observed, and this is most often accompanied by root rot, which compromises the plant's root system resulting in plant death.

Our knowledge of the pathology and host range of *R. solani* AG-2-2IIIB has accumulated over the years; however, very little is known about its ecology and the genetic structure of different populations at the field and regional scales. *R. solani* AG-2-2IIIB possesses an excellent saprophytic ability, and the production of long-lived "nutrient-independent propagules" called sclerotia (Hoitink et al. 1991) confers upon it the ability to survive for long periods in the absence of a suitable host plant. Reproduction is mostly asexual, and the vegetative structures, mycelia and sclerotia, are associated with infection and dispersal. Although sexual reproduction in some *R. solani* AGs is well documented (Ceresini et al. 2002; Lee and Rush 1983), field studies documenting basidiospore production of AG-2-2IIIB on soybean are lacking; therefore, the role that sexual reproduction plays in the disease cycle and in creating and maintaining diversity among populations of this subgroup currently is unknown.

Knowledge of the amount and distribution of genetic diversity within and among populations of different *R. solani* AGs is of practical significance, especially in making predictions about their evolutionary response to different disease management practices (McDonald and McDermott 1993). Moreover, an understanding of the genetic structure of the populations of different AGs or subgroups will provide important clues to the mating systems and reproductive modes that have shaped the populations over time. Studies on the population genetics of *R. solani* have been hampered mostly by difficulties in inducing the sexual stages of field isolates in vitro and the difficulty in distinguishing, in the absence of clamp connections, multinucleate homokaryons from heterokaryons (Cubeta and Vilgalys 1997). Nonetheless, the population structure of a number of AGs and subgroups, especially those associated with economically important crops, have been studied using different molecular markers. Using restriction fragment length polymorphic (RFLP) markers, Rosewich et al. (1999) provided evidence for sexual reproduction among populations of AG-1 IA recovered from six major rice-growing counties in Texas. However, using simple sequence repeat markers, Bernardes de Assis et al. (2008) found that the population structure of AG-1 IA isolates from soybean and rice in Louisiana was characterized by both sexual and asexual reproduction. A mixed reproductive system has been reported for AG-3 (Ferrucho et al. 2013; Ceresini et al. 2002), while populations of AG-8 are assumed to be predominantly clonal (MacNish et al. 1993). In addition, amplified fragment length polymorphism (AFLP) analysis of field isolates of AG-2-2IV and their single basidiospore progeny have provided evidence for both homothallic and heterothallic mating behaviors in this subgroup. Mating system and reproductive modes of AG-2-2IIIB is unknown, although Cubeta and Vilgalys (1997) suggested heterothallism and a mixed reproductive mode.

Diversity quantification and assessment in plant pathogenic fungi have, over the years, been accomplished using a variety of phenotypic and molecular markers, including vegetative compatibility groups, fungicide/antibiotic resistance, mating type, allozymes, RFLPs, random amplified polymorphic DNA (RAPD) and AFLPs (Brown 1996); however, microsatellites (SSRs) and single nucleotide polymorphisms (SNPs) recently have become the markers of choice (Milgroom 2015). According to Milgroom (2015), an ideal marker for population genetic studies should not be associated with a phenotype under selection, must be polymorphic enough to detect differences among the populations being studied, must be locus specific, must possess minimal homoplasy, must allow for the scoring of both homozygotes and heterozygotes (codominant), must be in linkage equilibrium, and must be amenable to repeatable, unambiguous scoring. While both SSR and SNP markers have many of the characteristics of an ideal marker, the use of SNPs for the study of genetic diversity among fungal populations is beginning to receive more attention given the advent of next generation sequencing technologies (NGS) that enable the simultaneous discovery and genotyping of thousands of markers in multiple individuals. Genotyping-by-sequencing (GBS) (Elshire et al. 2011) represents one approach for the simultaneous discovery and genotyping of SNPs, and its application in population genetic studies of fungi is well documented (Milgroom et al. 2014; Wilson et al. 2015).

Given the importance of AG-2-2IIIB as a pathogen of soybean, the lack of information about mating systems in this subgroup, as well as the lack of information on the diversity among field populations, we sought to exploit GBS as a tool to examine the population structure of AG-2-2IIIB isolates to enable us make inferences regarding the role of asexual and sexual reproduction in creating diversity and to provide insight into the evolutionary factors that shape the population structures of different field populations. Using populations of AG-2-2IIIB from

Illinois, Ohio, and Ontario, we conducted analysis to answer the following questions: (i) what is the genetic structure of the population of AG-2-2IIIB recovered from soybean seedlings across different geographical locations. Is the observed genetic structure consistent with that of a pathogen that reproduces sexually or asexually? (ii) What is the reproductive mode of the AG-2-2IIIB? Is it sexual, asexual, or is there evidence for a mixed reproductive system? Considering that members of this subgroup are considered to be largely clonal, we additionally sought for evidence of recombination in addition to detecting departures from random mating (iii) Are the populations of AG-2-2IIIB from Illinois, Ohio, and Ontario differentiated/genetically isolates, or do the isolates represent a large interbreeding population?

## Materials and Methods

### Fungal isolates

Sixty *R. solani* AG-2-2IIIB isolates recovered from symptomatic soybean seedlings were collected from Illinois ($n = 35$) , Ohio ($n = 8$) , and Ontario ($n = 17$) (Table 3.1). The Illinois isolates were collected from six counties in Illinois between 1991 and 2013. The Ohio isolates were collected by Dr. Anne Dorrance (Ohio State University, Columbus, OH) between 1998 and 2000, while the isolates from Ontario were collected by Albert Tenuta (Ontario Ministry of Agriculture and Food) from Merlin, Ontario and Rodney, Ontario in the summer of 2013. The Illinois and Ontario isolates had been previously characterized by our lab using conventional and molecular techniques as members of AG-2-2IIIB (data not shown). Although isolates from Ohio were reported to be AG-2-2IIIB, they were further characterized by PCR amplification with AG-2-2IIIB-specific primers (Carling et al. 2002) and by PCR-RFLP of the internal transcribed spacer (ITS) regions of the ribosomal genes as described by Guillemaut et al. (2003). Isolates were initially maintained on potato dextrose agar (PDA) amended with 25 mg/liter of rifampicin.

After five days of growth, isolates were prepared for long-term storage at -80°C by transferring five 5 mm agar plugs from PDA plates to 1.5 ml micro centrifuge tubes containing 850 µl of 15% glycerol.

**DNA extraction, SNP library construction, and genotyping**

For genomic DNA extraction, each isolate was grown at 25°C room temperature in 100 ml of potato dextrose broth (Difco Laboratories, Detroit, MI) for 14 days without shaking. Mycelia were harvested and DNA was extracted using FastDNA® spin kits (MP Biomedicals, Santa Ana, CA). DNA of each isolate was diluted to 40 ng/µl and confirmed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). SNP libraries were developed by slightly modifying the protocols described by Poland et al. (2012). A pilot restriction digest with four enzyme combinations (*PstI-HinP1I*, *PstI-BfaI*, *HindIII-HinP1I*, and *HindIII-BfaI*) initially was conducted on eight random samples to identify the best restriction enzyme combination for *R. solani*. Enzymes *PstI* and *HindIII* are rare cutters, while *HinP1I* and *BfaI* are common cutters. The *HindIII-HinP1I* combination was selected based on the absence of adapter dimers. For the 60 samples, the restriction-ligation reaction was performed in a 25 µl reaction volume containing 5 µl (200 ng) of genomic DNA, 2.5ul 10X NEB CutSmart buffer , 2.5ul 10 mM dATP , 0.1ul (2U) *HindIII*, 0.2ul *HinP1I* , 0.1ul concentrated T4 DNA ligase (40U), 0.5ul of 10uM Adapter2, and 14.1 ul molecular biology-grade water. Restriction digest was run at 37°C for 2 h followed by heat inactivation at 80°C for 15 min, while ligation reaction was done at 25°C for 2 h and a heat inactivation at 65°C for 20 min. DNA from barcoded libraries were pooled and cleaned using a 1:1 ratio of AmpureXP Beads (Beckman Coulter, CA, USA) to DNA, washed twice in 80% ethanol and resuspended in 15 µl of 10mM Tris elution buffer. PCR amplification of the purified pooled DNA was conducted in a 50 µl reaction

comprising 3 µl of DNA, 2 µl of Illumina primer mix (forward and reverse), 25 µl of Phusion High Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), and 20 µl of water. Cycle parameters for PCR amplification were: 98°C for 30 s, 18 cycles (98°C for 10 s, 68°C for 30 s, 72°C for 30 s), and 72°C for 5 min. Amplification products were subjected to a second cleanup as described above, followed by an examination of DNA size and concentration using an Agilent 2100 Bioanalyzer and Agilent DNA 7500 kit (Agilent Technologies Inc., CA, USA). Two separate libraries that were simultaneously constructed were diluted to 10 nM and pooled before submission for single-end, 100 bp sequencing using the Illumina HiSeq2000 sequencing system at the W.M. Keck Center (University of Illinois, Urbana, IL).

From the Illumina Fastq files obtained from sequencing run, sequence reads were matched to samples using the DNA barcode sequence. To call single nucleotide polymorphisms (SNPs), the consensus genome sequence of *R. solani* strain Rhs1AP (Cubeta et al. 2014) was downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) for use as a reference genome. However, as none of the reads aligned to the reference genome, SNPs were called using a Universal Network Enabled Analysis Kit (UNEAK) pipeline (Lu et al. 2013). Minor allele frequency was set at 0.05%, and SNPs were called at a minimum coverage cutoff of 0.5 (i.e. at least 50% non-missing data). Monomorphic SNPs, as well as SNPs with more than 10% missing data, were excluded before further analysis, and missing data was imputed to allele frequency. For data analysis, data were coded following the specified format for analyzing codominant genotypic data in GenAlEx 6.5 (Peakall and Smouse 2012).

**Genotype diversity and population differentiation**

Since *R. solani* AG-2-2-IIIB is considered to be functionally asexual, and populations are assumed to exhibit a clonal structure that is maintained from one generation to the next via asexual reproduction, we began diversity assessment by determining the genotypic diversity within each population. Genotypic diversity is a reflection of genotypic richness, or the number of multilocus genotypes (MLG) present in a population, and genotypic evenness. An MLG is a unique combination of alleles from multiple independent loci (Milgroom 1996). Multilocus genotypes were constructed using the function mlg.filter from the R package *poppr* (Kamvar et al. 2014). For this analysis, MLGs were collapsed with Nei's genetic distance using a distance threshold of 0.030 and the Unweighted Pair Group Method and Arithmetic Mean (UPGMA) as clustering algorithm.

Estimates of MLG tend to be biased upwards for samples with larger sizes, making comparison of genotypic richness for samples of unequal samples sizes invalid; therefore, the expected number of MLG (eMLG) adjusted to the smaller sample size was determined by rarefaction analysis (Grünwald et al. 2003), allowing for a comparison of genotypic richness among populations. Minimum spanning networks (MSN) were constructed to assess the genetic relatedness among MLGs for the Illinois population and for the three geographical regions combined. MLGs were generated using the imsn function in *poppr*, and Nei's distance was used to calculate the genetic distance between MLGs.

Using the non-clone-corrected data, indices of genotypic diversity for each population, including the Stoddart and Taylor's diversity index, $G_0$ (expressed as: $G_0 = 1/\sum p_i^2$ , where $p_i$ is the frequency of the *i*th genotype), the Simpson's diversity index, $\lambda$ (expressed as $\lambda = 1 - \sum p_i^2$, where $\sum p_i^2$ is the sum of the squared genotype frequencies), and the Shannon-Weiner index

($H'$), were determined using the *poppr* package in R. Clonal fraction, which is the proportion of fungal isolates in a sample originating from asexual reproduction, was calculated as 1-[(number of different genotypes/(total number of isolates)] (Zhan et al. 2003) for each population. The distribution of genotypes, otherwise referred to as genotypic evenness $E_5$, was calculated as the ratio of the number of abundant genotypes to the number of the rarer genotype (Grünwald et al. 2003). To compare diversity statistics among populations, confidence intervals for $G_0$, $\lambda$, and $E_5$ were obtained using the diversity_ci function of the *poppr* package in R. For downstream analyses, isolates having the same MLG were considered clones, and data were clone-corrected (using the clonecorrect function in *poppr* package) so that isolates with the same MLG were represented only once.

An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted to determine if the populations were genetically differentiated. First, we calculated $\Phi_{PT}$, an analogue of $F_{ST}$ that does not take into account variation within individual isolates, to determine if most of the genetic variation observed among the isolates was due to genotypic differences within or among populations. Next, we determined the overall population genetic differentiation, $F_{ST}$, which partitions the genetic diversity observed among all the individuals to diversity among the populations ($F_{ST}$), among individuals ($F_{IS}$), and within individuals ($F_{IT}$). For this analysis, the null and alternative hypotheses were:

$$H_0: F_{ST} = 0 \text{ (i.e. no genetic differentiation among populations)}$$

$$H_1: F_{ST} > 0 \text{ (i.e. populations are subdivided)}$$

Statistical significance of $F_{ST}$ was based on 9,999 permutations, and a significant genetic differentiation among populations was declared when $P \leq 0.05$. AMOVA and significance testing were conducted in GenAlEx 6.5. AMOVA was conducted on both clone-corrected and

non-clone-corrected data. Due to sample size reduction after clone correction, the Ohio population was not included in this analysis.

**Reproductive mode and population structure**

To address the question about the reproductive biology of *R. solani* AG-2-2IIIB, three approaches were employed to ascertain whether the populations are clonal or sexual. First, we conducted tests to determine associations among the SNP loci as an indication of whether or not the populations are in linkage disequilibrium (LD). The index of association, $I_A$, which is based on the variance in the number of alleles, *k*, at which two pairs of MLGs differ, is a useful metric for testing multilocus LD (Brown et al. 1980). Under conditions of random mating, the observed variance, $s_k^2$, should approach the expected variance, $\sigma_k^2$, producing an $I_A$ of zero (where $I_A = \frac{s_k^2}{\sigma_k^2} - 1$) (Milgroom 1996). However, the value of $I_A$ increases with the number of loci examined, making comparison among populations difficult; hence, a standardized index of association, $\bar{r}_d$, which is independent on the number of loci and can, as a result, permit comparison across data sets, is often preferred over $I_A$ for the estimation of the extent of LD. Estimates of $I_A$, and $\bar{r}_d$ were obtained and used to test the null hypothesis that the populations of AG-2-2IIIB isolates are sexually reproducing (i.e. are randomly mating). Significance testing of estimates of $I_A$ and $\bar{r}_d$ was done by 1,000 permutations, and *P*-values $\geq 0.05$ indicate no LD. Estimation of $I_A$ and $\bar{r}_d$ for both the uncorrected and clone-corrected data and significance testing of LD estimates were done using the *poppr* package in R. LD tests were not conducted on the Ohio population due to sample size reduction after clone-correction. The second approach involved using the branching patterns (topology) and lengths of phylogenetic trees as indicators of sexual reproduction. This is based on the assumption that the process of sexual recombination results in identical alleles with different evolutionary histories, resulting in incongruent

phylogenetic trees or trees that lack resolution (Burt et al. 1996; Milgroom 1996). Therefore, sexual populations should produce multifurcating branches with long tree lengths, while clonal populations should produce bifurcating trees with short tree lengths. Bootstrapped dendograms were calculated using the function aboot of the *poppr* package, and phylogenetic trees were constructed using the function plot.phylo in the R package *ape.* Bootstrap analysis was performed using 1,000 bootstrap replicates, and Nei's genetic distance was used for calculating genetic distances among isolates. The third approach involved determining the inbreeding coefficient, $F_{IS}$, for each population to test the hypothesis of mating among related individuals as a possible cause of deviation from the expectation of random mating (sexual reproduction). Mating systems in organisms that reproduce sexually can vary from strict inbreeding (homothallic) to predominantly outcrossing (homothallic). Populations that undergo inbreeding or mating among closely related individuals usually are characterized by a deficit of heterozygous loci compared to populations that are randomly mating due to sharing of alleles among individuals within the population. The inbreeding coefficient, which is expressed as "deficiency of heterozygotes relative to the expected heterozygosity under random mating" (Milgroom 2015), was calculated in ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). *P* values $\leq 0.05$ indicate a significant deviation from random mating.

Population structure or admixture has been recognized as a potential cause of LD. An admixed population can arise from a large migration event or when two genetically differentiated subpopulations are treated as a single undifferentiated population. Therefore, an assessment of population structure is necessary to help validate conclusions of lack of sexual recombination (or LD). The genetic structure of the combined Illinois, Ohio, and Ontario populations were assessed using both the uncorrected and the clone-corrected data. Population structure was examined by

conducting a discriminant analysis of principal component (DAPC) to detect admixed genotypes or individuals. DAPC is a multivariate analysis method that combines the strengths of principal component analysis (PCA) and discriminant analysis (DA) to investigate the genetic structure of populations by maximizing the between-population variability and minimizing the within-population variability (Jombart et al. 2010). Similar to PCA, DAPC does not rely on the assumptions of random mating (or Hardy-Weinberg equilibrium) or LD, and it can be applied to both sexually and asexually reproducing populations. For DAPC analysis, a cross validation analysis was conducted to determine the appropriate number of principal components to retain (based on mean squared error) that would explain most of the variability among the defined population. Cross validation and DAPC analysis were conducted using the *adegenet* package in R.

## Results

### Genotypic diversity and population differentiation

A total of 21 MLGs were identified among the 60 isolates recovered from Illinois, Ohio and Ontario. The number of MLGs varied across populations, with the ON population having the highest number (12), followed by IL (9) and OH populations (5) (Fig. 3.1). Two MLGs were shared among the three populations, while one MLG was shared between the Illinois and Ohio populations (Fig. 3.1). An examination of distribution of MLGs in Illinois (Fig. 3.2) revealed that nine MLGs were shared across five of the six counties from where the isolates were recovered. The single isolate representing Williamson County (IL_WI) had a unique MLG. DeKalb County (IL_DK) had the highest number of MLGs, which were four. Of these, one was shared with isolates from Whiteside County (IL_WS) and the other was shared with isolates from an unidentified County (IL_UN). Warren County (IL_WA) produced three MLGs, two of which

were shared with Champaign (IL_CH) and Whiteside (IL_WS) counties. Two MLGs were detected among isolates from Champaign County (IL_CH). Genotypic richness and overall genotypic diversity was highest for the Ontario population; however, evenness was similar in the Illinois and Ohio populations (Table 3.2). The Illinois and Ohio populations were comparable in terms of richness and genotypic diversity; however, the former had a lower genotypic evenness. Percent clonality was low for the Ontario and Ohio populations but high for the Illinois population.

AMOVA (Table 3.3) showed significant population differentiation between the Illinois and Ontario population for the uncorrected data ($\Phi_{PT} = 0.171; P = 0.002$); however, after clone correction, no genetic differentiation was observed between both populations ($\Phi_{PT} = 0.006; P = 0.385$). The variation between the Illinois and Ontario populations accounted for 1% of the total variance, while that within populations accounted for 99% (Table 3.3). From the estimates of the overall population genetic differentiation, $F_{ST}$, we found that for the clone-corrected data, 44% and 56% of the total variance were attributable to differences among individuals within populations and to differences within individuals, respectively.

**Reproductive mode and population structure**

Significance testing of the estimates of $I_A$, and $\bar{r}_d$ rejected the null hypothesis of sexual reproduction in the three populations for the uncorrected data ($P < 0.001$) and in the Illinois and Ontario populations for the clone-corrected data ($P < 0.001$) (Table 3.2). Results from the bootstrapped trees showed that the three populations did not cluster according to geographic origin; rather, each cluster observed comprised members from each population. Interestingly, phylogenetic analysis revealed multifurcating branches with longer terminal branch lengths for most members of the Ontario population, providing evidence for sexual reproduction in this

population. Inbreeding coefficient values for Illinois, Ohio, and Ontario were 0.443 ($P < 0.001$), 0.552 ($P = 0.005$), and 0.433 ($P < 0.001$), respectively.

DAPC clustering with the uncorrected data achieved a 95% overall reassignment of isolates to their prior groups; however, clone correction reduced the overall reassignment to 73%. These findings indicate the presence of admixed genotypes, which were defined based on posterior membership probabilities less than 90%. For the uncorrected data, 100, 75, and 96% of the genotypes comprising the Illinois, Ohio, and Ontario populations, respectively, were assigned to their prior population. With clone correction, membership probabilities dropped to 56%, 60%, and 91% for the Illinois, Ohio, and Ontario populations, respectively.

**Discussion**

This work provides the first in-depth assessment of the genetic structure of *R. solani* AG-2-2IIIB populations associated with seedling disease of soybean in North America. Our first objective was to assess the genetic structure of this subgroup to determine if the observed diversity is consistent with that of a sexual or recombining population. Given the lack of reports documenting the isolation of sexual structures in soybean fields, as well as our unsuccessful attempts to induce sexual reproduction in vitro, we hypothesized that field populations were mostly clonal. One of the hallmarks of asexual reproduction is low genotypic diversity (Milgroom 1996; McDonald and Linde 2002), which is a function of the number of unique genotypes (genotypic richness) and their frequencies (genotypic evenness). Similarly, an overrepresentation of MLGs and LD among loci are strong indicators of clonality (Tibayrenc et al. 1991). The results from this study suggest a mixed reproductive mode for the Ontario and Ohio populations and an asexual mode of reproduction for the Illinois population. A comparison of the eMLG, overall genotypic diversity, and clonal percentage revealed that the Ontario

population was the most genotypically diverse. Despite the high genotypic diversity observed for this population, we found significant associations among alleles at different loci for both the uncorrected and clone-corrected data, providing evidence for clonal reproduction. However, phylogenetic analysis revealed long terminal multifurcating branches for most of the members of this population. These findings provide evidence to support an asexual mode of reproduction that is also probably characterized by very little sexual reproduction. Currently, there is no information regarding the population genetic structure of *R. solani* AG-2-2IIIB from soybean-growing regions to allow for a comparison of genetic diversity or the role of sexual reproduction as a mechanism of genetic variation. However, isolates of AG-2-2IIIB recovered from turfgrass fields in Guelph, Ontario were found to be genetically diverse (Zheng et al. 2013). Similarly, Taheri and Tarighi (2012) found a high level of genetic diversity among AG-2-2IIIIB isolates recovered from diseased sugar beet plants in Iran. Unfortunately, none of these studies reported the occurrence of sexual structures in the fields from which these isolates were recovered. Interestingly, the production of sexual spores in sugar beet fields by isolates of AG-2-2IV have been reported (Toda and Hyakumachi 2006). Also, on fields planted to table beet in New York, AG-2-2 isolates causing foliar blight and root rot have been found to produce the sexual structures (Olaya and Abawi 1994). These observations altogether suggest a role for sexual reproduction as a source of genetic variation in AG-2-2, especially in the populations of the different subgroups.

Our rejection of the null hypothesis of sexual reproduction for the Ontario population may be due to several reasons. First, "founder effect" coupled with inbreeding might have had a significant impact. Inbreeding coefficient estimates from our results indicate that considerable inbreeding might be occurring in this population. If the population was started by very few

related individuals with fixed differences in alleles, association among alleles will still be detected even though individuals within the population were randomly mating. Second, given the high dependence of LD on sample size, we may not have had enough statistical power to detect sexual recombination due to the limited number of samples evaluated in this study. To better understand the role of sexual reproduction as a mechanism of variation in the populations of *R. solani* AG-2-2IIIIB associated with soybean seedling disease, there is the need to continue searching for sexual reproductive structures in nature to corroborate inferences based on population genetics.

Despite sample size differences between the Illinois ($n = 35$) and Ohio ($n = 8$) populations, genotypic richness was comparable between these two populations. However, an examination of the clonal fraction within each population revealed a higher clonal percentage for the Illinois population than for the Ohio population. Interestingly, we also found that MLGs identified in Illinois were shared among populations from different counties separated in time and space (Fig 3.2). These findings, coupled with our rejection of the null hypothesis of no LD among loci, lend support to our hypothesis of a predominantly clonal population structure for the Illinois population. Differences in the genotypic diversities observed between the Illinois and Ontario populations may be explained by crop species diversity. Host diversity has been suggested as a potential factor influencing the genetic structure of fungal populations, including populations of *R. solani* AG-2-2IIIB (Taheri and Tarighi 2012) and those of *Magnaporthe grisea* (Tredway et al. 2005; Douhan et al. 2011).While the isolates comprising the Illinois population were recovered from fields planted mostly to soybean and corn, the fields from which the Ontario isolates were recovered are planted to a wide variety of crops, including soybean, corn, wheat, tobacco, vegetable crops, and fruit trees (Albert Tenuta, personal communication). In

addition to host diversity as a possible explanation for the differences in genotypic diversity and reproductive mode, differences in fungal life cycles across different geographies may be an artifact of region-based differences in the biology and mode of dispersal of infective structures (Taylor et al. 1999).

A surprising finding from this study is the significantly low levels of population differentiation among the populations examined. Partitioning the overall genetic variation among the isolates of the Illinois and Ontario population revealed that 99% of the genetic diversity was within populations and only 1% was between the populations. Population subdivision was further supported by the lack of separation of the different populations in the UPGMA tree (Figs 3.3 and 3.4) and the finding that certain MLGs were shared across different geographies (populations) and across different counties for the Illinois population. Moreover, DAPC revealed the presence of admixed genotypes, especially after clone-correction (Fig. 3.5). Our results do not agree with those of Taheri and Tarighi (2012) who found significant differentiation among AG-2-2IIIB isolates from turfgrass and the lack of sharing of genotypes across geographic locations. Lack of population differentiation and significant amount of gene and genotype flow among geographically separated populations of *R. solani* AG-3 and AG-1A have been reported and attributed to the human-mediated movement of infected plant material and the movement of sclerotia via irrigation water (Bernardes-de-Assis et al. 2009; Ferrucho et al. 2013). We propose that the low level of genetic differentiation of the populations in this study may be attributed to genotype flow or long distance dispersal of asexual propagules, resulting in genetic similarity among isolates from different regions. Genotype flow may have occurred via anthropogenic activities that move soil, such as the movement of contaminated vehicles and machinery, or via sclerotia dispersal, which may have been mediated by rainfall, irrigation, or drainage.

The importance of this study relates to how the information on the population genetic structure of *R. solani* AG-2-2IIIB can be exploited for improving the management of the seedling disease it incites on soybean. The unavailability of commercial soybean cultivars marketed as resistant to Rhizoctonia root and hypocotyl rot makes the application of seed treatment fungicides the most common management method. Fungicide resistance development is of growing concern with an over-reliance on fungicide application as a sole management strategy. Although most of the fungicide seed treatment products used to control seedling diseases of soybean contain active ingredients with multiple modes of action, a few of these products may sometimes contain a single active ingredient belonging to a chemistry class that is at a high risk of selecting resistant genotypes. The biology of the fungus, in addition to the fungicide breadth of activity, is another factor that can greatly determine the potential of a pathogen to develop new genotypes that can adapt to changing environments. Our results indicate that populations of *R. solani* AG-2-2IIIB are characterized by either a mixed reproductive system or an asexual form of reproduction, and that the exchange of genotypes among geographically separated populations is a predominant factor that shapes population structure. According to the risk model framework proposed by McDonald and Linde (2002) to predict the evolutionary potential of a pathogen on the basis of its population genetic structure, pathogens with a mixed reproductive mode pose the greatest risk. This is because the process of asexual reproduction maintains and increases the frequency (via selection) of well adapted genotypes created through the process of sexual reproduction. Therefore, there is the need to exercise caution when applying fungicides and the need for the continued search for sources of resistance in the soybean germplasm to increase available options for disease management.

This study focused on populations of *R. solani* from two states in the U.S. and one province in Canada. Sampling design and sample size may have been inadequate to make an overall assessment of diversity and to detect random mating among members of each population. There is the need for more extensive studies elucidating the population genetic structure of populations from similar and additional locations. Extensive sampling may be required to improve the statistical power for detecting sexual recombination and to clarify the role of gene and genotype flow as an evolutionary force shaping the population structure of this subgroup, especially on fields planted to soybean.

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## Tables

**Table 3.1.** Origin and year of isolation of *Rhizoctonia solani* AG-2-2IIIB isolates recovered from soybean seedlings in Illinois, Ohio, and Ontario.

| Isolate ID | Isolate ID used | Isolate origin | Year of isolation |
|---|---|---|---|
| 12Rs41 | 12Rs41 | Williamson, IL | 2012 |
| 211_1b | 211_1b | Champaign, IL | 2012 |
| 42210_b | 42210_b | Unknown, IL | 2013 |
| 42210_c | 42210_c | Unknown, IL | 2013 |
| 42304_b | 42304_b | Unknown, IL | 2013 |
| 42304_c | 42304_c | Unknown, IL | 2013 |
| 42304_g | 42304_g | Unknown, IL | 2013 |
| 42304_h | 42304_h | Unknown, IL | 2013 |
| BF09476 | BF09476 | Champaign, IL | 1991 |
| DK_10 | DK_10 | DeKalb, IL | 2013 |
| DK_11 | DK_11 | DeKalb, IL | 2013 |
| DK_13 | DK_13 | DeKalb, IL | 2013 |
| DK_14 | DK_14 | DeKalb, IL | 2013 |
| DK_15a | DK_15a | DeKalb, IL | 2013 |
| DK_15B | DK_15B | DeKalb, IL | 2013 |
| DK_16 | DK_16 | DeKalb, IL | 2013 |
| DK_18 | DK_18 | DeKalb, IL | 2013 |
| DK_19 | DK_19 | DeKalb, IL | 2013 |
| DK_3a | DK_3a | DeKalb, IL | 2013 |
| DK_3b | DK_3b | DeKalb, IL | 2013 |
| DK_4a | DK_4a | DeKalb, IL | 2013 |
| DK_4b | DK_4b | DeKalb, IL | 2013 |
| DK_6a | DK_6a | DeKalb, IL | 2013 |
| DK_6b | DK_6b | DeKalb, IL | 2013 |
| DK_7 | DK_7 | DeKalb, IL | 2013 |
| DK_8 | DK_8 | DeKalb, IL | 2013 |
| ER_15 | ER_15 | Whiteside, IL | 2013 |
| ER_19a | ER_19a | Whiteside, IL | 2013 |
| ER_19b | ER_19b | Whiteside, IL | 2013 |

**Table 3.1 (cont.)**

| Isolate ID | Isolate ID used | Isolate origin | Year of isolation |
|---|---|---|---|
| ER_2 | ER_2 | Whiteside, IL | 2013 |
| ER_4 | ER_4 | Whiteside, IL | 2013 |
| K_ILSO2_3_25a | K_ILSO2_3_25a | Waren, IL | 2013 |
| K_ILSO2_3_25b | K_ILSO2_3_25b | Waren, IL | 2013 |
| K_ILSO2_3_25c | K_ILSO2_3_25c | Waren, IL | 2013 |
| Rs1039 | Rs1039 | Champaign, IL | 2000 |
| 91_1 | 91_1 | Ohio | 1998-2000 |
| NT_12 | NT_12 | Ohio | 1998-2000 |
| NT_13 | NT_13 | Ohio | 1998-2000 |
| NT_14 | NT_14 | Ohio | 1998-2000 |
| NT_15 | NT_15 | Ohio | 1998-2000 |
| NT_15_Jan | NT_15_Jan | Ohio | 1998-2000 |
| NT_18 | NT_18 | Ohio | 1998-2000 |
| PL_1 | PL_1 | Ohio | 1998-2000 |
| WONS 2013-8-7 | MC12 | Merlin, Ontario | 2013 |
| WONS 2013-8-8 | MC10 | Merlin, Ontario | 2013 |
| WONS 2013-8-6 | MC03 | Merlin, Ontario | 2013 |
| PDONS 2013-8-6 | MC06 | Merlin, Ontario | 2013 |
| PDONS 2013-8-3 | MC07 | Merlin, Ontario | 2013 |
| WONS 2013-8-5 | MC14 | Merlin, Ontario | 2013 |
| PDONS 2013-12-6 | MC16 | Rodney, Ontario | 2013 |
| PDONS 2013-8-5 | MC05 | Merlin, Ontario | 2013 |
| PDONS 2013-8-1 | MC15 | Merlin, Ontario | 2013 |
| WONS 2013-12-3 | MC01 | Rodney, Ontario | 2013 |
| PDONS 2013-8-4 (Storage albert) | MC04 | Merlin, Ontario | 2013 |
| WONS 2013-8-3 | MC11 | Merlin, Ontario | 2013 |
| PDONS 2013-8-8 | MC09 | Merlin, Ontario | 2013 |
| WONS 2013-8-1 | MC13 | Merlin, Ontario | 2013 |
| PDONS 2013-8-7 | MC08 | Merlin, Ontario | 2013 |
| WONS 2013-8-2 | MC02 | Merlin, Ontario | 2013 |
| WONS 2013-8-4 (Storage Albert) | MC17 | Merlin, Ontario | 2013 |

**Table 3.2**. Genotypic diversity and linkage disequilibrium in *Rhizoctonia solani* AG-2-2IIIB isolates from Illinois, Ohio, and Ontario

| Indices/Metrics | Ontario | Illinois | Ohio |
|---|---|---|---|
| ***N*** | 17 | 35 | 8 |
| **Genotypic diversity** | | | |
| Multilocus genotypes (MLGs) ~ (richness) | 12 | 9 | 5 |
| eMLG (expected richness) | 8.03 | 4.84 | 5 |
| Shannon's diversity (H') | 2.36 | 1.84 | 1.49 |
| Stoddard and Taylor's genotypic diversity (G) | 9.32 | 3.91 | 4.00 |
| Simpson's diversity (λ) | 0.92 | 0.76 | 0.80 |
| $E_5$ (evenness) | 0.86 | 0.67 | 0.87 |
| Clonal fraction (1-(MLG/N) | 0.29 | 0.74 | 0.38 |
| **Linkage disequilibrium** | | | |
| $I_A^{\,u}$ | 179.92*** | 401.84*** | 348.22*** |
| $\bar{r}_d^{\,u}$ | 0.106*** | 0.233*** | 0.227*** |
| $I_A^{\,c}$ | 132.87*** | 151.88*** | - |
| $\bar{r}_d^{\,c}$ | 0.079*** | 0.088*** | - |

[u] indicates estimates for uncorrected data
[c] indicates estimates for clone-corrected data
*** indicates significance at $P < 0.001$

**Table 3.3.** Analysis of molecular variance of *Rhizoctonia solani* AG-2-2IIIB isolates from Illinois and Ontario

| Source of variation | Uncorrected data | | | | Clone-corrected data | | | |
|---|---|---|---|---|---|---|---|---|
| | **df** | **% of total** | **Fixation Statistic** | ***P* value** | **df** | **% of total** | **Fixation Statistic** | ***P* value** |
| $\Phi_{PT}$ | | | | | | | | |
| Among population | 1 | 17.14 | 0.171 | 0.002 | 1 | 1 | 0.006 | 0.385 |
| Within population | 50 | 82.86 | | | 19 | 99 | | |
| $F_{ST}$ | | | | | | | | |
| Among population | 1 | 12.47 | 0.125 | 0.001 | 1 | 0 | 0.004 | 0.346 |
| Among individuals within population | 50 | 33.08 | 0.378 | 0.001 | 19 | 44 | 0.437 | 0.001 |
| Within individuals | 52 | 54.45 | 0.456 | 0.001 | 21 | 56 | 0.440 | 0.001 |

## Figures

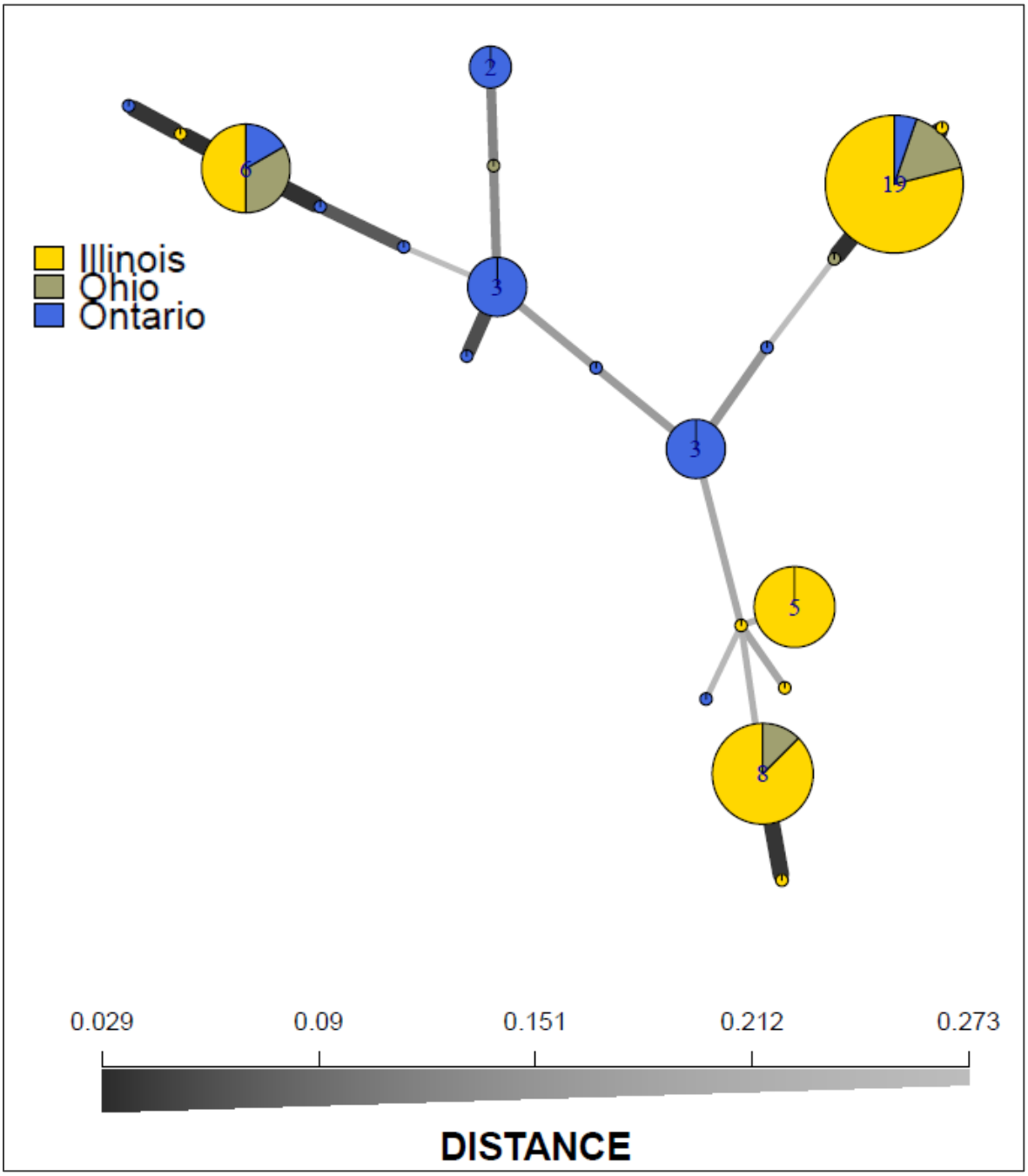


**Fig. 3.1.** Minimum spanning network showing the multilocus genotypes (MLGs) observed in the Illinois, Ohio, and Ontario populations of *Rhizoctonia solani* AG-2-2-IIIB and the relationship among the MLGs. Each node/circle represents a different MLG, and the number inside each node represents the frequency of that MLG. Node color represents population membership. Line size and color is proportional to Nei's genetic distance indicated in the scale bar.

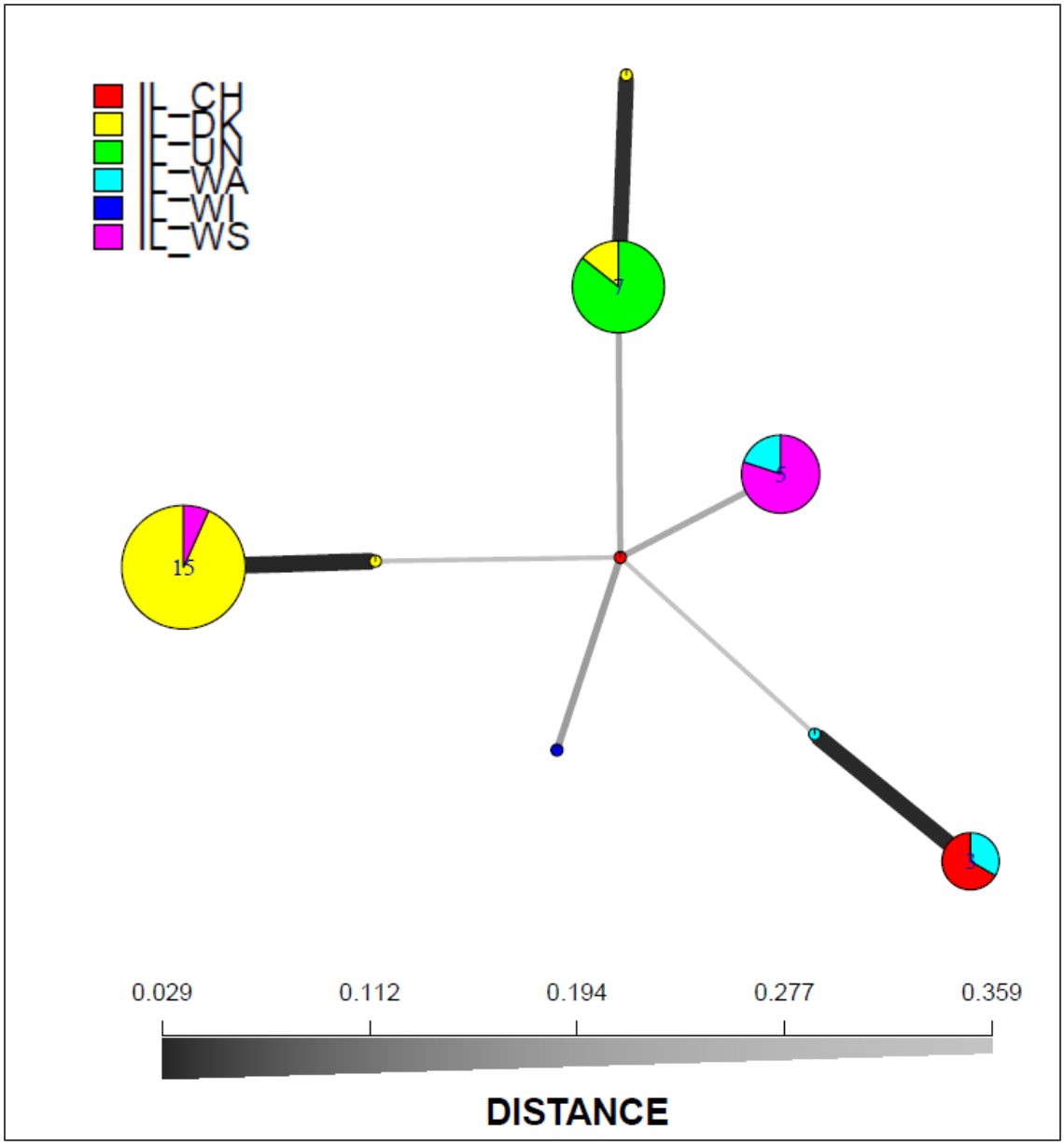


**Fig. 3.2.** Minimum spanning network showing the distribution and relationship between the multilocus genotypes (MLGs) observed in the five counties in Illinois where *Rhizoctonia solani* AG-2-2-IIIB were recovered. Each node/circle represents a different MLG, and the number inside each node represents the frequency of that MLG. Node color represents population membership. Line size and color is proportional to Nei's genetic distance indicated in the scale bar.

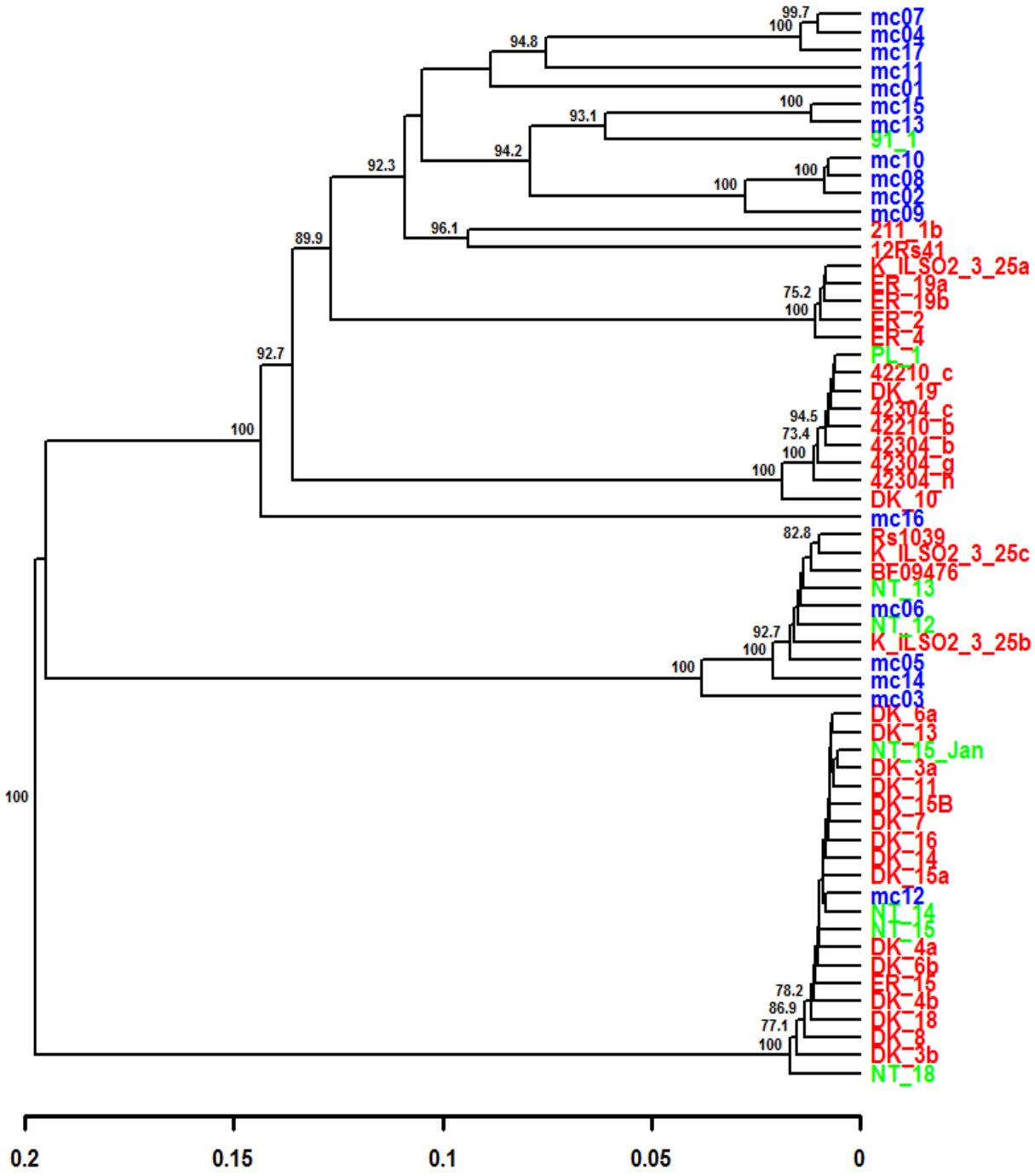


**Fig. 3.3.** Unweighted pair group method with arithmetic mean (UPGMA) dendogram of *Rhizoctonia solani* AG-2-2IIIB isolates (uncorrected data) from Illinois (red node labels), Ohio (green node labels), and Ontario (blue node labels) based on Nei's genetic distance. Bootstrap support values greater than 70% using 1000 bootstrap samples are shown at the nodes.

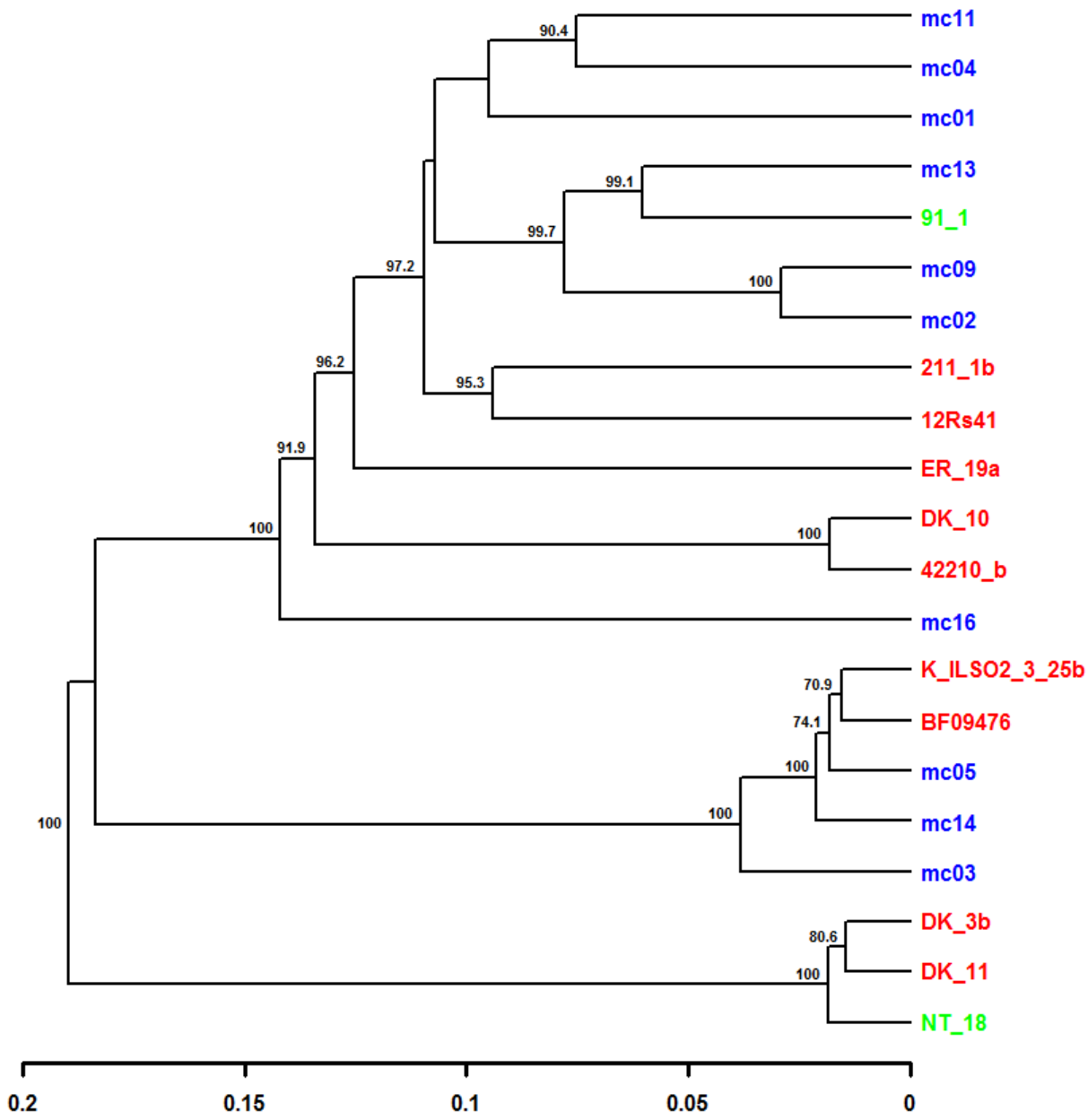


**Fig. 3.4**. Unweighted pair group method with arithmetic mean (UPGMA ) dendogram of *Rhizoctonia solani* AG-2-2IIIB isolates (clone-corrected data) from Illinois (red node labels), Ohio (green node labels), and Ontario (blue node labels) based on Nei's genetic distance. Bootstrap support values greater than 70% using 1000 bootstrap samples are shown at the nodes.

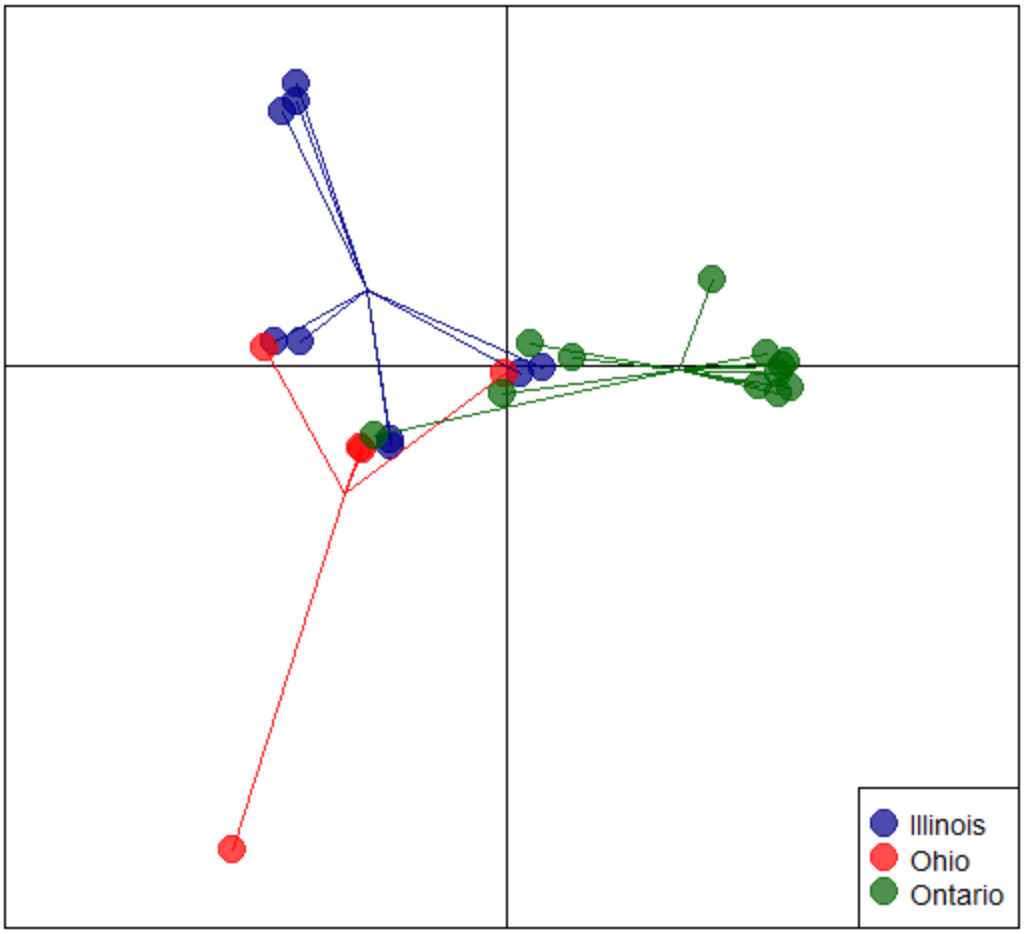


**Fig. 3.5.** Discriminant analysis of principal components scatter plot showing the first and second principal components discriminating *Rhizoctonia solani* AG-2-2-IIIB populations from Illinois, Ohio, and Ontario. Lines are included to connect members of the same population.

# CHAPTER 4: SENSITIVITY OF *RHIZOCTONIA SOLANI* TO SUCCINATE DEHYDROGENASE INHIBITOR AND DEMETHYLATION INHIBITOR FUNGICIDES


## Abstract

Seedling diseases of soybean caused by *Rhizoctonia solani* can be managed with seed-applied fungicides that belong in different chemistry classes. To provide a benchmark for assessing possible decline in sensitivities to these fungicide classes, *R. solani* isolates collected prior to the year 2001 were evaluated for their sensitivities to succinate dehydrogenase inhibitor (SDHI) fungicides (penflufen and sedaxane) and demethylation inhibitor (DMI) fungicides (ipconazole and prothioconazole). The effective concentration of each fungicide that reduced mycelial growth by 50% ($EC_{50}$) was determined in vitro and compared with those of isolates recovered after 2011 from diseased soybean plants across different soybean growing regions in the United States and Canada. Most isolates collected prior to 2001 (87.5%) and after 2011 (98.8%), were sensitive ($EC_{50} = 0.01 < EC_{50} < 0.1$ µg/ml) to penflufen. However, for sedaxane, 20% of the pre-2001 isolates and 38.8% of the isolates collected after 2011 were observed to be only moderately sensitive ($EC_{50} = 0.1 < EC_{50} < 1$ µg/ml). For the DMI fungicides, all isolates tested, regardless of when they were isolated, had $EC_{50}$ values $\geq 0.1$ µg/ml, with the exception of one isolate that was collected after 2011 that had an ipconazole $EC_{50}$ value of 0.078 µg/ml. For all four active ingredients, variation in sensitivity was observed within and among the different anastomosis groups comprising both groups of isolates. Isolates collected after 2011 which had varying in vitro sensitivities were further evaluated for in vivo sensitivity to the four fungicides in the greenhouse. In vitro fungicide sensitivity was not always correlated with fungicide efficacy in the greenhouse. None of the four fungicides evaluated provided complete protection

in greenhouse experiments; however, plant stand generally was significantly greater when seeds were treated with a fungicide compared to the non-treated, inoculated control. Overall, our results showed that sensitivity to the fungicide classes tested has decreased in comparison to the baseline *R. solani* population, but control of seedling disease caused by *R. solani* was still achieved regardless of in vitro sensitivity. Based on this research, continued monitoring of fungicide sensitivities of *R. solani* populations should occur to determine if sensitivities become further reduced in the future.

## Introduction

*Rhizoctonia solani* (syn. *Thanatephorus cucumeris*), a ubiquitous necrotrophic fungus, causes economically important diseases of soybean worldwide. In the north central United States, seedling diseases caused by *R. solani*, including seed decay, pre- and post-emergence damping off, hypocotyl rot, and root rot, can suppress yield (Doupnik 1993), and yield losses of up to 48% have been reported on small research plots in Iowa (Tachibana 1968). Although considered to be members of the same species, isolates of *R. solani* exhibit marked genetic diversity, leading to their classification into anastomosis groups (AGs) and subgroups within certain AGs. To date, fourteen AGs (AGs 1 – 13 and AG-B1) have been identified (Carling et al. 1999; Carling 1996; Ogoshi 1987). Five AGs have been associated with soybean seedling disease, which are AG-2-2IIIB (Fenille et al. 2002; Liu and Sinclair, 1991; Muyolo et al. 1993), AG-3 (Nelson et al. 1996), AG-4 (Bolkan and Ribeiro 1985; Fenille et al. 2002; Muyolo et al. 1993; Nelson et al. 1996; Ploetz et al. 1985; Rizvi and Yang 1996; Zhao et al. 2005), AG-5 (Nelson et al. 1996), AG-7 (Baird et al. 1996), and AG-11 (Carling et al. 1994). Isolates of AG-2-2IIIB are highly aggressive on soybean roots (Muyolo et al. 1993; Nelson et al. 1996) and are generally more aggressive than the hypocotyl-infecting isolates of AG-4 (Fenille et al. 2002; Muyolo et al. 1993;

Zhao et al. 2005). AG-5 isolates have been reported to be pathogenic on soybean seedlings (Nelson et al. 1996; Zhao et al. 2005) and adult plants (Nelson et al. 1996), while isolates of AG-3 (Nelson et al. 1996), AG-7 (Baird et al. 1996), and AG-11 (Carling et al. 1994) cause only minimal symptoms on soybean seedlings.

With the unavailability of commercial soybean cultivars marketed for resistance (Bradley et al., 2001), soybean growers in the north central U.S. rely mostly on seed treatment fungicides to protect soybean seedlings against *R. solani* infections. Two of the seed treatment fungicides commonly used to manage seedling diseases of soybean caused by *R. solani* belong to two different fungicide chemistry classes: demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI). The DMIs inhibit fungi by targeting C14-demethylase, an important enzyme required for the biosynthesis of sterols (Koller 1988; Ragsdale 1975). DMI fungicides are effective against a wide range of fungal organisms and offer preventive, curative, and eradicative functions (Kuck et al. 2012; Mueller et al. 2013). SDHIs inhibit respiration in target organisms by inhibiting the activity of the succinate dehydrogenase complex, which is a large complex of the respiratory chain involved in ATP generation (Avenot and Michailides 2010; Yankovskaya et al. 2003). Although earlier generations of the SDHIs, such as carboxin, provided excellent protection against basidiomycetes (Hewitt 1998), newer members of this class possess a broad spectrum of activity against a wide range of fungal pathogens (Sierotzki and Scalliet 2013) and are thereby becoming more important as a disease control option in the crop protection industry. Members of these two fungicide chemistries exhibit specificity in their mode of action and have the potential to select for resistant genotypes. Many fungal pathogens have been reported to show reduced sensitivities to active ingredients in both fungicide classes under field and laboratory conditions (Avenot and Michailides 2007; Bayles et al. 2000; De Waard et

al. 1986; Karaoglanidis et al. 2000; Keon et al. 1991; Mavroeidi and Shaw 2005; Miller and Gubler 2003).

Penflufen and sedaxane, which belong to the SDHI fungicide class, and ipconazole and prothioconazole, which belong to the DMI group, are four important active ingredients of seed treatment fungicides used for controlling seedling diseases of soybean caused by *R. solani*. Generally, seed treatment fungicides marketed for managing soybean seedling diseases, including those caused by *R. solani*, are either formulated as mixtures of different active ingredients with the potential to control a broad range of seedling pathogens, or sold as solo products with specificity for a particular pathogen. Despite the potential risk for resistance development with the single-site inhibitor solo products, there are currently no reports documenting the sensitivity of soybean-infecting isolates of *R. solani* to these active ingredients in historical and recently-collected isolates. In addition to the fungicide mode of action, genetic variation within the pathogen population is another factor that can generally increase the risk of fungicide resistance. Given the biology of *R. solani* as a functionally asexual organism (Adams 1996), fungicide resistance is not expected to be a problem; however, resistance to the quinone outside inhibitors (QoI), another single-site inhibitor, has been reported in AG-1 populations of *R. solani* causing foliar diseases of rice and soybean (Olaya et al. 2012). Although resistance to the SDHI and DMI classes of fungicides has not been reported for *R. solani*, Fungicide Resistance Action Committee (2016) reports a medium to high resistance risk for the SDHIs and a medium resistance risk for the DMIs. Therefore, to guard against resistance development in the populations of *R. solani* treated with the currently used SDHI and DMI seed treatments, it is pertinent that the sensitivities of historical isolates be compared to sensitivities of recently-collected isolates. This would establish acceptable levels of fungicide sensitivities which can

then be used in subsequent monitoring programs to determine possible shifts in the sensitivity of *R. solani* populations. The objectives of our research were to (i) identify possible shifts in sensitivity to each fungicide class and (ii) determine, using in vivo assays, the effectiveness of the selected fungicides in managing isolates showing a range of in vitro sensitivities.

## Materials and Methods

### Collection, isolation and storage of *R. solani* isolates

Historical isolates of *R. solani* evaluated for sensitivities were recovered from diseased soybean and sugar beet plants between 1988 and 2000. Two isolates from Illinois (Liu and Sinclair 1991) were included as part of the historical group. Other isolates of this group included those recovered from Ohio (Dorrance et al. 2003), which were obtained from Dr. Anne Dorrance at the Ohio State University (Wooster, OH), and those recovered from the Red River Valley in Minnesota (MN) and North Dakota (ND), which were obtained from Dr. Carol Windels at the University of Minnesota (Crookston, MN) (Liu and Sinclair 1992). Because of the soybean-sugar beet (*Beta vulgaris*) rotation system in the Red River Valley of MN and ND, and the observation that sugar beet is a host to the AGs and subgroups of *R. solani* that also infect soybean, isolates recovered from sugar beet were included for evaluation in this study. This group of historical isolates represented two important AGs (Table 4.1) previously reported to be highly aggressive on soybean (Liu and Sinclair 1991; Muyolo et al. 1993; Nelson et al. 1996).

To assess the current sensitivities of *R. solani* populations to fungicides, isolates recovered after 2011 were evaluated. The isolates were collected from Illinois, Arkansas, and Ontario, Canada. Isolates from Arkansas and Ontario were originally collected by Dr. John Rupe (University of Arkansas, Fayetteville, AR) and Dr. Albert Tenuta (Ontario Ministry of Agriculture and Food), respectively. These isolates are curated in Dr. Ahmad Fakhoury's

laboratory (Southern Illinois University, Carbondale, IL). Isolates from Illinois were collected from soybean seedlings with sunken reddish-brown lesions on hypocotyls, root rot and/or damping off symptoms. These isolates were recovered from eight counties between 2012 and 2014. Roots of all seedlings were washed under running tap water to remove soil particles, disinfected with 1% NaOCl, and rinsed in sterile distilled water. Cut sections from lesions of symptomatic plants were placed on Ko and Hora medium (Ko and Hora 1971) and incubated at room temperature for 24 to 48 h. Hyphal tips from colonies resembling *Rhizoctonia* were transferred to potato dextrose agar (PDA) amended with rifampicin to minimize bacterial contamination. All isolates were identified based on cellular nuclear number using a safranin O staining technique (Matsumoto et al. 1932), cultural and morphological characteristics, hyphal anastomosis reactions (Kronland and Stanghellini 1988), and sequence comparison of the rDNA ITS regions with the ITS4 and ITS5 primer (White et al. 1990). Only isolates determined to be multinucleate and identified as *R. solani* were selected for assessments. All isolates collected after 2011 were identified to five AGs (Table 4.1).

Plugs (5 mm diameter) of selected isolates on PDA were placed in 1.5 ml micro centrifuge tubes containing 850 µl of 15% glycerol and stored at -80°C for long-term storage. Hyphal tips from cultures were transferred to new antibiotic-amended PDA plates containing sterile table beet seeds and incubated 10 days at 25°C. Colonized beet seeds were dried under a sterile flow hood for 48 h, placed in 2.0 ml Nalgene cryogenic vials (Sigma-Aldrich, St. Louis, Missouri), and stored at 4°C. New cultures from 4°C storage were used for all experiments to avoid using genetically unstable isolates that might have arisen due to repeated sub-culturing (Sneh and Adams 1996).

**In vitro sensitivity assay**

A total of 40 pre-2001 isolates and 80 isolates collected after 2011 were evaluated for their sensitivity to four active ingredients (a.i.) belonging to two different fungicide classes: penflufen (98.72% a.i.) and sedaxane (95% a.i.) for the SDHIs, and prothioconazole (97.7% a.i.) and ipconazole (40.7% a.i.) for the DMIs. A pilot study, using 10 randomly selected isolates from both groups of isolates, was initially conducted to determine the fungicide concentrations required for each fungicide class (data not shown). Appropriate volumes, based on percent a.i., of each fungicide were dissolved in acetone to obtain stock solutions, and serial dilutions were carried out to obtain the following concentrations for the two chemistry classes: 0.001, 0.01, 0.1, and 1 µg/ml for the SDHIs, and 0.01, 0.1, 1, and 10 µg/ml for the DMIs.

Using a sterile cork borer, 5 mm diameter plugs were excised from the edge of 4-day old cultures on rifampicin-amended PDA, inverted, and transferred to the center of 100 mm × 15 mm petri dishes containing fungicide-amended media. There were two replicate plates for each isolate-fungicide concentration combination, and inoculated fungicide-amended plates were incubated at 25°C in continuous light. Non-amended PDA plates served as controls. At 48 h after inoculation, mycelial diameter at opposite axes was measured, and the average diameter was calculated. Diameter of the agar plug was subtracted from the average mycelial diameter, and the percent growth inhibition for each isolate by fungicide combination was determined using the formula

$$((\text{diameter of control} - \text{diameter of fungicide amended plate}) / (\text{diameter of control})) \times 100\%.$$

The effective concentration that resulted in 50% mycelial growth inhibition ($EC_{50}$) for each isolate was calculated using linear interpolation (Wise et al. 2008).

Due to the large size of the experiment and space constraints, sensitivity of all isolates to all four fungicides could not be tested in a single trial; therefore, for each individual fungicide assayed, isolates were evaluated in batches or trials, and each batch comprised 10 isolates. To minimize variability across batches, an internal check, isolate BF09476, was assayed for its sensitivity to each of the four active ingredients in 10 separate pilot trials, and the mean $EC_{50}$ value and 95% confidence interval was determined for all 10 trials (Wong and Wilcox 2002). Only main experimental trials in which the $EC_{50}$ value of the internal check fell within the 95% confidence interval obtained from the pilot trials were considered valid and were included for statistical analysis. The experiment was arranged as a randomized complete block design and repeated in a second run, with each run serving as a block. Each experiment comprised all isolates, which were evaluated in a total of 12 batches. $EC_{50}$ data were log-transformed to ensure normal distribution and homogeneity of residuals before analysis in SAS (version 9.4; SAS institute Inc., Cary, NC). Data were analyzed using the mixed model procedure (PROC MIXED) with trial and AG-type as fixed factors and isolate nested within AG as a random factor. Mean differences among AGs were determined using the PDMIX800 macro (Saxton 1998) in SAS (version 9.4). Using the Pearson correlation procedure (PROC CORR) in SAS (version 9.4), associations between $EC_{50}$ values of active ingredients belonging to the same chemistry class were determined. For the AG-2-2 and AG-4 isolates, combined $EC_{50}$ values obtained from the pre-2001 isolates and isolates collected after 2011 were further analyzed to determine significant differences in mean $EC_{50}$ values within each AG. To compare degree of sensitivity among isolates, the following in vitro sensitivity scale was adopted: highly sensitive ($EC_{50} < 0.01$ µg/ml), sensitive ($0.01 < EC_{50} < 0.1$ µg/ml), moderately sensitive ($0.1 < EC_{50}$ 1 µg/ml), and less sensitive ($EC_{50} \geq 1$ µg/ml).

**In vivo sensitivity assay**

Greenhouse assays were conducted on a subset of *R. solani* isolates to determine the effectiveness of the four fungicides in controlling seedling disease of soybean. The isolates were selected and categorized on the basis of the $EC_{50}$ values obtained in the in vitro assay (Table 2). Inocula for all isolates were prepared as described by Paulitz and Schroeder (2005) with slight modifications. Briefly, 250 ml of sorghum seeds were soaked in 250 ml distilled water in 500 ml flasks and autoclaved for 30 min at 137.9 kPa and at 120°C. After 24 h, a second autoclaving was carried out after which flasks were allowed to cool. Beet seeds colonized with the isolates were recovered from storage at 4°C and maintained on PDA for 4 days. Inoculation of autoclaved sorghum seeds was carried out by transferring five 15-mm diameter agar plugs from the 4-day-old PDA culture of each isolate into separate flasks containing autoclaved sorghum seeds. Inoculated sorghum seeds were incubated at room temperature and shaken every other day to ensure uniform colonization of seeds. After two weeks of incubation, inoculated sorghum seeds were dried in a laminar flow hood for 3 days, after which they were stored in paper bags and maintained at 4°C before use.

Seeds of soybean cultivar Williams 82 were treated with slurries of four fungicide seed treatments: penflufen at 2.13 g a.i./100 kg seed (Evergol Prime; Bayer CropScience, Research Triangle Park, NC), sedaxane at 1.09 g a.i./100 kg seed (Vibrance; Syngenta Crop Protection, Greensboro, NC), ipconazole at 1.14 g a.i./100 kg seed (Vortex; Bayer CropScience), and prothioconazole (Proline; Bayer CropScience). Plastic pots (12.7 cm diameter) were half-filled with a layer of steam-pasteurized 2:1 sand:silt loam soil, covered with 2 g of inoculum, and then covered with about 5 cm layer of soil before planting, and each pot received 10 seeds. Two separate experiments were conducted for the SDHI and DMI fungicides. The experiment was set

up as a randomized complete block in a split plot arrangement with four replications. Isolate served as the whole plot and fungicide seed treatment as the subplot. Fungicide seed treatment consisted of two active ingredients and a non-treated but inoculated control for both SDHI and DMI fungicide class. The experiment, which was conducted in the greenhouse under 14 h photoperiod and at 24 ± 3°C, was repeated in a second trial. At 18 days after planting, plants were evaluated for stand, dried root weight, and root and hypocotyl disease severity using a 0 to 5 scale modified from Nelson et al. (1996) as follows: 0 = no lesion on root or hypocotyl; 1 = lesions < 2.5 mm on hypocotyl and ≤ 20% of roots with lesions or rot symptoms; 2 = lesions 2.5 to 5 mm on hypocotyl and 20-40% of roots with lesions or rot symptoms; 3 = lesions > 5 mm on hypocotyl and 40-60% of roots with lesions or rot symptoms; 4 = lesions girdling entire hypocotyl and 60-80% of roots with lesions or rot symptoms; and 5 = plant dead, and/or no roots, or > 80% of roots with lesions or rot symptoms. The disease severity rating for each subplot unit was converted to a disease severity index (DSI) using the formula: $DSI = \frac{\sum_{i=1}^{5} s_i p_i}{5 \times N}$, where *s* is the disease score for each plant, *p* is the number of plants with disease score, and *N* is the total number of plants in each subplot unit. Tests for normality and homogeneity of residuals were conducted, and Box-Cox transformations (Box and Cox 1964) were carried out on each dependent variable when necessary. Statistical analysis was carried using PROC MIXED in SAS (version 9.4). To determine if trials could be pooled, a test of homogeneity of variance of both trials was conducted, and when heterogeneity was not significant, data were pooled. Comparison of least-square means was conducted with Fisher's protected least significant difference (LSD) test at alpha = 0.05, using the PDMIX800 macro (Saxton 1998) in SAS (version 9.4).

## Results

### In vitro sensitivity of pre-2001 isolates

The $EC_{50}$ values obtained with the DMIs were found to be consistently higher than those of the SDHIs. For example, the respective minimum and maximum $EC_{50}$ values obtained for the SDHI fungicides were 0.007 µg/ml and 0.155 µg/ml for penflufen, and 0.026 µg/ml and 0.215 µg/ml for sedaxane (Fig. 4.1). In contrast, for the DMIs, 0.349 µg/ml and 3.162µg/ml were the minimum and maximum mean $EC_{50}$ values for ipconazole, respectively, while prothioconazole had a 0.200 µg/ml minimum and a 3.665 µg/ml maximum (Fig. 4.2).

**SDHIs**: For penflufen and sedaxane, the analysis of variance (ANOVA) for the $EC_{50}$ produced a significant effect of AG ($P < 0.0001$). However, trial and the interaction between trial and AG were not significant (Table 4.3). For penflufen, the 27 AG-2-2 isolates had a significantly higher mean $EC_{50}$ of 0.056 µg/ml, with a range of 0.016µg/ml to 0.155 µg/ml. $EC_{50}$ values for the 13 AG-4 isolates ranged from of 0.007 µg/ml to 0.035 µg/ml, with a mean of 0.017 µg/ml. For sedaxane, the $EC_{50}$ values of the AG-2-2 isolates ranged from 0.029 µg/ml to 0.215 µg/ml with a mean of 0.089 µg/ml, while the AG-4 isolates had a significantly lower mean $EC_{50}$ of 0.051 µg/ml with $EC_{50}$ values ranging from 0.026 µg/ml to 0.119 µg/ml (Table 4.4). Based on the range of $EC_{50}$ values obtained, all isolates could be grouped as either highly sensitive ($EC_{50} < 0.01$ µg/ml) or sensitive ($0.01 < EC_{50} < 0.1$ µg/ml) to penflufen, while the response to sedaxane was either sensitive or moderately sensitive ($0.1 < EC_{50} < 1$ µg/ml). A significant ($P < 0.0001$) positive correlation ($r = 0.38$) was observed between the sensitivity to penflufen and sedaxane.

**DMIs:** For ipconazole, there were significant ($P < 0.0001$) differences between the mean $EC_{50}$ values obtained for the AG-2-2 and AG-4 isolates (Table 4.4). Isolates of AG-2-2 resulted

in a significantly lower mean $EC_{50}$ value of 1.338 µg/ml compared to the AG-4 isolates which had a mean $EC_{50}$ value of 1.894 µg/ml (Table 4.4). For prothioconazole, main effects of AG, trial, and trial by isolate interactions were not significant (Table 4.3). The mean $EC_{50}$ value for the AG-2-2 isolates was 1.497 µg/ml, while that of the AG-4 isolates was 1.809 µg/ml (Table 4.4). Based on the $EC_{50}$ values obtained for both active ingredients, all isolates could be categorized as either moderately sensitive or less sensitive ($EC_{50} \geq 1$ µg/ml). A significant ($P < 0.0001$) positive correlation ($r = 0.50$) was observed between the sensitivity to ipconazole and prothioconazole.

**In vitro sensitivity of the post-2011 isolates**

For the overall sensitivity to the SDHI and DMI fungicides, a similar trend as seen with the pre-2001 isolates was observed for the post-2011 isolates. The SDHIs produced $EC_{50}$ values lower than those of the DMIs. For the SDHIs, the respective minimum and maximum $EC_{50}$ values for penflufen were 0.014 µg/ml and 0.244 µg/ml, while those for Sedaxane ranged from 0.022 µg/ml to 0.282 µg/ml. For the DMIs, the minimum and maximum $EC_{50}$ values for ipconazole were 0.078 µg/ml and 3.434 µg/ml, respectively, while prothioconazole produced a minimum of 0.239 µg/ml and a maximum of 6.802 µg/ml.

**SDHIs**: For both penflufen and sedaxane, there were significant differences among AGs; however, the effect of trial and the interactions between trial and AG were not significant (Table 4.3). For penflufen, the AG-2-2 isolates not only had the widest range of $EC_{50}$ values, but they also had the highest mean $EC_{50}$ value of 0.054 µg/ml, although this was not significantly different from the means of the AG-3 (0.041 µg/ml), AG-7 (0.047 µg/ml) and AG-11 (0.040 µg/ml) isolates (Table 4.4). For sedaxane, the AG-7 isolates had the highest mean $EC_{50}$ value of 0.162 µg/ml, which was significantly higher than those of the other four AGs. A wide range of

$EC_{50}$ values were observed for the AG-2-2, AG-7, and AG-11 isolates but not for the AG-3 or AG-4 isolates (Table 4.4). A significant ($P < 0.0001$) positive correlation (r = 0.40) between the sensitivities to penflufen and sedaxane was observed.

**DMIs**: For the pre-2001 isolates, a significant AG effect and a non-significant effect of trial and trial by AG interaction was observed for ipconazole and prothioconazole (Table 4.3). For ipconazole, the mean $EC_{50}$ value obtained for the AG-11 isolates (1.797 µg/ml) was the highest, followed by isolates of AG-2-2 (1.143 µg/ml), AG-7 (0.495 µg/ml), AG-4 (0.298 µg/ml), and AG-3 (0.199 µg/ml). The $EC_{50}$ values of the AG-2-2 and AG-11 isolates were highly variable and ranged as follows: 0.148 µg/ml to 3.434 µg/ml for the AG-2-2 isolates, and 0.913 µg/ml to 2.490 µg/ml for the AG-11 isolates. With the exception of one AG-4 isolate, which had an $EC_{50}$ value of 0.078 µg/ml, all other isolates of AG-4 and isolates of AG-3 and AG-7 had $EC_{50}$ values that fell between 0.1 µg/ml and 1 µg/ml, and were therefore considered moderately sensitive to ipconazole. Highly variable $EC_{50}$ values were similarly observed for prothioconazole, especially for the AG-2-2 (0.275 µg/ml to 6.802 µg/ml), AG-7 (0.334 µg/ml to 2.733 µg/ml), and AG-11 (0.283 µg/ml to 1.791 µg/ml) isolates. All AG-3 and AG-4 isolates were moderately sensitive ($0.1 < EC_{50} < 1$ µg/ml) to prothioconazole, while isolates of AG-2, AG-7, and AG-11 could be classified into the moderately sensitive ($0.1 < EC_{50} < 1$ µg/ml) and less sensitive categories ($EC_{50} \geq 1$ µg/ml). None of the isolates evaluated were considered highly sensitive or sensitive to prothioconazole. $EC_{50}$ values of ipconazole and prothioconazole were not significantly correlated ($P = 0.11$; r = 0.09).

**Comparison of sensitivities of the pre-2001 and post-2011 isolates of AG-2-2 and AG-4 for the four fungicides**

**AG-2-2 isolates**: For penflufen, the mean $EC_{50}$ values of the pre-2001 isolates were not significantly different from those of the post-2011 isolates (Fig. 4.3); however, for sedaxane, the mean $EC_{50}$ of the post-2011 isolates was significantly greater than that of the pre-2001 isolates. The pre-2001 isolates had a higher mean $EC_{50}$ for Ipconazole (Table 4.4), which was significantly different from that for the isolates recovered after 2011 (Fig. 4.3). For prothioconazole, compared to the pre-2001 isolates, a significantly higher mean $EC_{50}$ was observed for the isolates recovered after 2011.

**AG-4 isolates**: The mean $EC_{50}$ value of the post-2011 group was numerically higher but not significantly different from those of the pre-2001 group (Fig.4. 4) for penflufen and sedaxane. Ipconazole and Prothioconazole treatments, however, showed contrasting results: the pre-2001 group had significantly higher mean $EC_{50}$ values than the post-2011 group.

**In vivo sensitivity assay**

**SDHI fungicides**: Four isolates, selected on the basis of their in vitro sensitivity levels expressed in terms of $EC_{50}$ values, were evaluated in the greenhouse to determine the effectiveness of the two SDHI fungicides in controlling root and hypocotyl rot of soybean. Since we observed that for this fungicide class, all isolates could not be grouped into the four sensitivity categories adopted for the in vitro assay, we selected isolates that fell on the low, medium and high spectrum of the sensitivity range for each fungicide class (Table 4.2). For statistical analysis, DSI was transformed to $DSI' = DSI^{-1}$ and stand count data were transformed to $SC' = SC^3$ to ensure normal distribution and homogeneity of residuals. Therefore, the ANOVA presented for DSI and stand count are for transformed data. From the

ANOVA, significant main and interaction effects were observed for some of the dependent variables (Table 4.5). For all three dependent variables (stand count, dried root weight, and disease severity), the main effect of isolate was not significant (Table 4.5), while that of fungicide was highly significant ($P < 0.0001$). A significant isolate by fungicide interaction was observed for stand count and dried root weight only (Table 4.5), implying that the response of the selected isolates varied depending on the seed treatment applied. For isolates 1 and 4, which exhibited high in vitro sensitivity to penflufen and sedaxane, respectively, the dried root weights and stand counts obtained when either fungicide seed treatments was applied were significantly higher than the non-treated control (Table 4.6). For isolate 2, which showed moderate sensitivity to both penflufen and sedaxane in vitro, sedaxane gave significantly higher dried root weights and stand counts over the non-treated control; however, dried root weights and stand count resulting from penflufen treatment was not significantly different from the means of the non-treated control. Penflufen and sedaxane did not significantly increase root weights and stand counts for the highly insensitive isolate 3. Compared to the non-treated control, lower disease severity indices were generally observed with penflufen and sedaxane, regardless of the isolate.

**DMI fungicides**: Six isolates which varied in their in vitro sensitivity levels to either ipconazole or prothioconazole (Table 4.2) were selected for evaluation in the greenhouse. Selection criterion was similar to that adopted for the SDHI fungicides. To ensure normal distribution and homogeneity of residuals, the following transformations were carried out: $DSI' = DSI^{-1}$ for disease severity index; $SC' = SC^{2}$ for stand count; and $RW' = RW^{0.5}$ for dried root weight. Results from the ANOVA revealed significant main effects of isolate and fungicide for all three dependent variables and a significant isolate by fungicide interaction for DSI only (Table 4.5). For the ipconazole-sensitive isolate 5, ipconazole seed treatment did not

result in a lower DSI. Compared to the non-treated control, ipconazole produced a lower DSI for the ipconazole-moderately-sensitive isolate 6 and the ipconazole-less-insensitive isolate 7. DSI obtained from prothioconazole seed treatment for the prothioconazole-moderately-sensitive isolate 9 was not significantly different from the non-treated control, while significantly lower DSI was obtained for both the prothioconazole-sensitive isolate 8 and the prothioconazole-less-sensitive isolate 10. Regardless of isolate, significantly higher stand counts and root weights were obtained with ipconazole and prothioconazole treatments.

**Discussion**

The sensitivity of *R. solani* to several fungicide classes has been studied in various in vivo and in vitro assays (Amaradasa et al. 2014; Barnes et al. 1990; Blazier and Conway 2004; Campion et al. 2003; Carling et al. 1990; Goll et al. 2014; Kataria et al. 1991a, b; Li et al. 2014; Martin et al. 1984a, b; Meyer et al. 2006; Zhang et al. 2009). Unfortunately, varied results were obtained within and among the different AGs tested, and the majority of the fungicides evaluated are currently not in use for managing diseases caused by this pathogen. Moreover, with the exception of Barnes et al. (1990), none of these studies clearly indicated previous exposures of the isolates tested to the fungicides evaluated. The historical isolates selected in this study were recovered prior to 2001 when all four active ingredients evaluated had not been registered for use as a seed treatment on soybean or sugar beet. Penflufen and sedaxane became available to the U.S. soybean industry in 2012. Ipconazole received approval as a soybean seed treatment from the U.S. Environmental Protection Agency (EPA) in 2008, while prothioconazole became available as a seed treatment in 2013.

Based on the sensitivities of the two groups of isolates, our results indicate that all isolates examined were more effectively controlled by the SDHI fungicides. For penflufen, a greater

percentage of the pre-2001 (87.5 %) and post-2011 (98.8%) isolates were classified under the sensitive category, and as high as 80% and 61% of the pre-2001 and post-2011 isolates, respectively, were sensitive to sedaxane (Table 4.7). For both SDHI fungicides, we observed that the mean and median $EC_{50}$ values of the post-2011 isolates were slightly higher than those of the historical isolates, suggesting that a slight shift towards reduced sensitivity to these fungicides might be occurring. Contrary to what was observed with the SDHIs, a greater percentage of both groups of isolates showed reduced sensitivity to the DMI fungicides. For example, 77.5% of the pre-2001 isolates and 47.5% of the post-2011 isolates were less sensitive to ipconazole. For prothioconazole, as high as 70% of the pre-2001 isolates and 56.3% of the post-2011 isolates were less sensitive. These observations indicate that both the historical isolates and those recovered after 2011 exhibited reduced sensitivity to the DMI than to the SDHI fungicides. According to Hewitt (1998), sensitivity shifts are more often observed with fungicidal compounds that have been in use for many decades (Hewitt 1998). The DMI fungicides are reported to have been available for use since the mid-1970s (Mueller et al. 2013), although they they were not used on soybean until their approval as a section 18 emergency product for the management of soybean rust in the mid-2000s. Given the potential for cross-resistance between members of this fungicide group (Fungicide Resistance Action Committee 2016), the likelihood of a previous exposure of our historical and post-2011 isolates to other DMI fungicides cannot be completely ruled out, and this might explain the observed reduced sensitivity of both groups of isolates to the DMIs. Nevertheless, shifts in sensitivity of the post-2011 isolates to both DMI fungicides were evident by higher mean and median $EC_{50}$ values than those of the pre-2001 isolates (Fig. 4.2).

Due to the genetic diversity of *R. solani*, an evaluation of the sensitivity of the different *R. solani* AGs that cause seedling diseases of soybean to currently used seed treatment fungicides will be important in making seed treatment recommendations, provided the predominant AGs in any one field is known. We found that penflufen provided strong activity towards the AG-2-2 (Fig. 4.3) and AG-4 isolates (Fig. 4.4) of the historical and post-2011 groups. Although for penflufen, the mean $EC_{50}$ value of the pre-2001 AG-4 isolates was numerically lower than that of the AG-4 isolates of the post-2011 group (Fig. 4.4), the range of $EC_{50}$ values obtained for all AG-4 isolates in both groups fell within the sensitive category ($0.01 < EC_{50} < 0.1$). There were no isolates representative of AG-3, AG-7, and AG-11 in the pre-2001 group, but the results obtained from the post-2011 group confirm the excellent activity of penflufen towards these AGs. Even though sedaxane belongs in the same fungicide chemistry class as penflufen, similar patterns of sensitivity among and within AGs were not observed for sedaxane (Tables 4.4 and 4.7). Variable sensitivity levels (ranging from sensitive to moderately sensitive) were obtained for all AGs of the pre-2001 and post-2011 isolates, with the exception of the post-2011 AG-3 and AG-4 isolates which, based on the $EC_{50}$ values, can be considered sensitive (Table 4.4). Our results with both penflufen and sedaxane agree with those of other authors who evaluated the sensitivity of different *R. solani* AGs to members of the SDHI fungicide chemistry class. For example, Goll et al. (2014) evaluated the sensitivity of *Rhizoctonia* species recovered from Europe to sedaxane and reported a low baseline sensitivity for isolates of AG-3PT (0.022 µg/ml), AG-3TB (0.043 µg/ml), AG-4 (0.028 µg/ml), and AG-11 (0.020 µg/ml). Carboxin, an older member of the SDHI class, has also been shown to possess strong activity towards different AGs (Kataria et al. 1991b; Martin et al. 1984a). Martin et al. (1984a) reported an $EC_{50}$ of 0.31 mg/liter for AG-2

isolates and 0.01 mg/liter for isolates of AG-4, while Kataria et al. (1991b) found that AG-7 isolates were highly sensitive, followed by AG-4 and AG-2-2 isolates.

Variable responses to the DMI fungicides were observed for both the pre-2001 and post-2011 isolates. Similar variability in sensitivity has been reported for triadimefon (Martin et al. 1984a), triticonazole (Amaradasa et al. 2014), bromuconazole, difenoconazole, and tebuconazole (Meyer et al. 2006), hexaconazole (Carling et al. 1990), and triflumizole (Csinos and Stephenson 1999). Based on our sensitivity scale, the pre-2001 and post-2011 isolates were either moderately or less sensitive to the DMI fungicides, with the exception of one post-2011 AG-4 isolate (EV_6) that exhibited sensitivity to ipconazole based on an $EC_{50}$ value of 0.078 µg/ml. Barnes et al. (1990) found that AG-4 isolates recovered from peanut and cowpea were sensitive to three DMI fungicides, diniconazole (mean $EC_{50}$ = 0.028 mg/liter), cyproconazole (mean $EC_{50}$ = 0.056 mg/liter), and tebuconazole (mean $EC_{50}$ = 0.166 mg/liter), although these isolates had never been previously exposed to this fungicide chemistry. A surprising finding from this study was that ipconazole and prothioconazole exhibited good activity towards the post-2011 AG-4 isolates but weak activity towards the pre-2001 AG-2-2 and AG-4 isolates and the post-2011 AG-2-2 isolates (Table 4.4). Although the historical isolates evaluated in this study are assumed to have been recovered before the commercial use on soybean of these two DMI fungicides, it is noteworthy to mention that for this group of isolates, all five AG-4 isolates and 13 of the 27 AG-2-2 isolates were recovered from the Red River Valley of Minnesota and North Dakota where sugar beet is a major rotational crop with soybean. Aside from *R. solani* AG-2-2 and AG-4, several different fungal pathogens, such as *Cercospora beticola* (Kerr and Weiss 1990; Miller et al. 1994; Weiland and Koch 2004), are known to infect sugar beet, and it is not unlikely that other DMI fungicides might have been used to control these pathogens in the past, resulting in an exposure

of *R. solani* isolates and the observed reduced sensitivity of the pre-2001 isolates to the fungicides we evaluated.

The goal of the in vivo fungicide evaluation conducted in this study was to determine if reduced fungicide sensitivity in the in vitro assays translated into reduced fungicide efficacy when applied as seed treatments. For both fungicide classes, we found that, in most cases, isolates exhibiting high or moderate sensitivity to certain fungicides in the in vitro assays were effectively controlled by the respective fungicides in the greenhouse. For example, isolates 1 and 4, which, in our in vitro assays, were highly sensitive to penflufen and sedaxane, respectively, were effectively controlled by both fungicides in the greenhouse assay. The significant fungicide × isolate interactions observed for stand count and dried root weight for the SDHI fungicides implied that fungicide efficacy was isolate-dependent. Isolate 3 was the only isolate against which neither penflufen nor sedaxane improved stands or root weights. This isolate was also less sensitive to these two fungicides in the in vitro assay based on the $EC_{50}$ values obtained for both fungicides. Our results indicate that although this isolate was able to produce disease symptoms on soybean roots and hypocotyls, as shown by a significantly higher disease severity index compared to the treated plants, it does not cause seed or root rot. Therefore, reduced virulence in this isolate may be correlated with its inability to be controlled by these two fungicides in petri dish assays. Isolate 3 is an AG-2-2IIIB isolate, and although virulence differences exist among members of this group (Dorrance et al. 2003), a correlation between virulence and fungicide sensitivity in *R. solani* has not been reported. Isolate 2 was moderately sensitive to both penflufen and sedaxane in our in vitro studies, but in the greenhouse, this this isolate was only effectively controlled by sedaxane. Martin (1984a, b) similarly found that certain *R. solani* and *R. zeae* isolates showing moderate sensitivity to PCNB and carboxin in in vitro assays were not

effectively controlled by these fungicides on tall fescue (*Festuca arundinacea*) in the greenhouse.

For the DMI fungicides, protection was evident in significantly higher stand counts regardless of isolate. Although root weights of non-treated plants were numerically lower for all isolates, no effect of fungicide was observed. It appears, therefore, that the DMI fungicides are very effective at protecting the seeds to ensure germination and emergence but not as effective in protecting the roots. Fungicide seed treatments are generally applied to soybean to protect against infection by soil-borne and seed-borne pathogens during the early stages of growth, and the efficacy of seed treatments in preventing stand losses has been demonstrated in greenhouse and field experiments (Bradley 2008; Dorrance et al. 2003; Urrea et al. 2013). However, as seed treatments are mostly retained around the cotyledon region, with little or none remaining around the root zone as the plants develop (Dorrance et al. 2003), protection of soybean roots from any invading soil-borne pathogen is expected to decline with plant age. Isolates 5 and 9, which were respectively considered to be sensitive to ipconazole and moderately sensitive to prothioconazole, had disease severity indices that were not significantly different from the non-treated control. However, ipconazole treatment resulted in significantly higher stand counts and root weights for isolate 5, while prothiconazole treatment resulted in significantly higher stand counts for isolate 9. These observations indicate that subjective assessments, such as disease severity ratings, may not be an appropriate measure for evaluating fungicide effectiveness in greenhouse or field studies.

In conclusion, our results indicate that *R. solani* isolates are more sensitive to the SDHI class of fungicides than to the DMI fungicides, and that considerable variation in fungicide sensitivity exist within and among AGs of this fungus. In vitro assays suggest sensitivity shifts to all four

fungicides, therefore necessitating the need to adopt other management options that may reduce future fungicide usage. Although our results from in vitro assays indicate reduced sensitivity to the DMI fungicides, results from greenhouse assays conducted suggest that protection in the field will be acceptable. While none of the fungicides evaluated provided complete disease control in the greenhouse assays, significantly higher stand counts of plants from treated seed compared to plants from non-treated seed further confirms the effectiveness of seed treatments in improving stand establishment and reducing the risk of total crop failure in the presence of disease risk factors. Lastly, our results indicate that reduced in vitro fungicide sensitivity may not always correlate with disease control in the field.

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## Tables

**Table 4.1.** Origin and number of isolates within each anastomosis group of *Rhizoctonia solani* evaluated for sensitivity to penflufen, sedaxane, ipconazole and prothioconazole

| Isolate ID | Year collected | Origin | Anastomosis group (AG) |
|---|---|---|---|
| BF09476 (65L-2) | 1991 | Illinois | AG-2-2IIIB |
| RS 1018(RS 159)[a] | Pre-2000 | Ohio | AG-2-2IIIB |
| PL_1 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_0 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_12 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_30 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_9 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_15 | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_11 | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_13 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_14 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_39 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| NT_18 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| 91_1 AD | Pre-2001 | Ohio | AG-2-2IIIB |
| 90_3_22 | 1990 | Clay County, MN | AG-4 |
| 18_s_21 | 1988 | Wilken County, MN | AG-2-2 |
| 95_16_2 | 1995 | ND | AG-2-2 |
| 18_s_22 | 1988 | MN | AG-2-2 |
| 95_3_6 | 1995 | Polk County, MN | AG-4 |
| 87_4_70 | 1987 | MN | AG-2-2 |
| 95_5_19 | 1995 | Polk County, MN | AG-4 |
| 86_71_6 | 1986 | Marshall County, MN | AG-2-2 |
| 88_11_3 | 1988 | Pembina County, ND | AG-4 |
| 18_S_26B | 1988 | MN | AG-2-2 |
| 87_24_4A | 1987 | MN | AG-2-2 |
| 86_72_7 | 1986 | Polk County, MN | AG-2-2 |
| 86_34_8 | 1986 | Traill County, ND | AG-4 |
| 87_18_2 | 1987 | Walsh County, ND | AG-4 |
| 95_16_6A | 1995 | Pembina County, ND | AG-4 |
| 95_8_12 | 1995 | Pembina County, ND | AG-4 |
| 87_12_23 | 1987 | Pembina County, ND | AG-4 |
| 88_12_2 | 1988 | Polk County, MN | AG-4 |
| 7_S_44 | 1988 | Wilken County, MN | AG-2-2 |

[a]Isolate was collected by Dr. Sally Miller (Ohio State University) but curated in Dr. Darin Eastburn's laboratory (University of Illinois, Urbana-Champaign)

**Table 4.1 (cont.)**

| Isolate ID | Year collected | Origin | Anastomosis group (AG) |
|---|---|---|---|
| 86_49_11 | 1986 | MN | AG-2-2 |
| 18_T_9 | 1988 | Polk County, MN | AG-2-2 |
| 93_29_6 | 1993 | MN | AG-2-2 |
| ST_10 | 1988 | Polk County, MN | AG-4 |
| 87_36_4 | 1987 | ND | AG-2-2 |
| E_O_20 | 1987 | Roseau County, MN | AG-4 |
| 95_4_19 | 1995 | Polk County, MN | AG-4 |
| K_ARSO2_1_20 | 2013 | Arkansas | AG-11 |
| K_ARSO2_2_5 | 2013 | Arkansas | AG-7 |
| K_ARSO2_1_7 | 2013 | Arkansas | AG-7 |
| K_ARSO2_1_6 | 2013 | Arkansas | AG-7 |
| K_ARSO2_1_9 | 2013 | Arkansas | AG-7 |
| K_ARSO2_1_8 | 2013 | Arkansas | AG-7 |
| BVT_18 | 2013 | St. Clair, IL | AG-11 |
| S_P_19b | 2013 | Pike, IL | AG-11 |
| S_P_19a | 2013 | Pike, IL | AG-11 |
| K_4_18b | 2013 | St. Clair, IL | AG-11 |
| 12SDSa | 2013 | IL | AG-11 |
| 12RS52 | 2012 | Champaign, IL | AG-11 |
| ER_4 | 2013 | Whiteside, IL | AG-2-2IIIB |
| ER_19b | 2013 | Whiteside, IL | AG-2-2IIIB |
| ER_15 | 2013 | Whiteside, IL | AG-2-2IIIB |
| ER_19a | 2013 | Whiteside, IL | AG-2-2IIIB |
| DK_8 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_19 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_15a | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_13 | 2013 | DeKalb, IL | AG-2-2IIIB |
| K_ILSO2_3_25c | 2013 | Warren, IL | AG-2-2IIIB |
| 42210_b | 2013 | IL | AG-2-2IIIB |
| DK_6a | 2013 | DeKalb, IL | AG-2-2IIIB |

[a]Isolate was collected by Dr. Sally Miller (Ohio State University) but curated in Dr. Darin Eastburn's laboratory (University of Illinois, Urbana-Champaign)

**Table 4.1 (cont.)**

| Isolate ID | Year collected | Origin | Anastomosis group (AG) |
|---|---|---|---|
| DK_4a | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_16 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_3b | 2013 | DeKalb, IL | AG-2-2IIIB |
| 42304h | 2013 | Unknown, IL | AG-2-2IIIB |
| DK_14 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_10 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_7 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_11 | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_3a | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_15b | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_4b | 2013 | DeKalb, IL | AG-2-2IIIB |
| DK_6b | 2013 | DeKalb, IL | AG-2-2IIIB |
| K_ILSO2_3_25a | 2013 | Warren, IL | AG-2-2IIIB |
| 42210_c | 2013 | IL | AG-2-2IIIB |
| K_ILSO2_2_13c | 2013 | Warren, IL | AG-2-2IIIB |
| 211_1 PDA | 2012 | Champaign, IL | AG-2-2IIIB |
| 12RS41 | 2012 | Williamson, IL | AG-2-2IIIB |
| 211_1a KH | 2012 | Champaign, IL | AG-2-2IIIB |
| 211_1b KH | 2012 | Champaign, IL | AG-2-2IIIB |
| IL 2014a | 2014 | Champaign, IL | AG-2-2IIIB |
| IL 2014b | 2014 | Champaign, IL | AG-2-2IIIB |
| 248_3b KH | 2012 | Jackson, IL | AG-3 |
| C_far_500_3 | 2013 | Champaign, IL | AG-3 |
| C_far_500_10a | 2013 | Champaign, IL | AG-3 |
| C_far_500_6 | 2013 | Champaign, IL | AG-3 |
| EV_3 | 2013 | Jackson, IL | AG-4 |
| BVT_28 | 2013 | St. Clair, IL | AG-4 |
| EV_6 | 2013 | Jackson, IL | AG-4 |
| BVT_3 | 2013 | St. Clair, IL | AG-4 |
| BVT_11 | 2013 | St. Clair, IL | AG-4 |

[a]Isolate was collected by Dr. Sally Miller (Ohio State University) but curated in Dr. Darin Eastburn's laboratory (University of Illinois, Urbana-Champaign)

**Table 4.1 (cont.)**

| Isolate ID | Year collected | Origin | Anastomosis group (AG) |
|---|---|---|---|
| BVT_16 | 2013 | St. Clair, IL | AG-7 |
| EV_19 | 2013 | Jackson, IL | AG-7 |
| EV_7 | 2013 | Jackson, IL | AG-7 |
| BVT_20 | 2013 | St. Clair, IL | AG-7 |
| WONS 13_8_7 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_8 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_6 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_6 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_3 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_5 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_12_6 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_5 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_12_3 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_4 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_3 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_8 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_1 | 2013 | Ontario | AG-2-2IIIB |
| PDONS 13_8_7 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_12_1 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_2 | 2013 | Ontario | AG-2-2IIIB |
| WONS 13_8_4 | 2013 | Ontario | AG-2-2IIIB |
| ONSO2_14 | 2012 | Ontario | AG-2-2IIIB |
| ONSO2_18 | 2012 | Ontario | AG-2-2IIIB |
| ONSO2_15 | 2012 | Ontario | AG-2-2IIIB |
| ONSO2_13 | 2012 | Ontario | AG-2-2IIIB |
| ONSO2_16 | 2012 | Ontario | AG-2-2IIIB |
| ONSO2_17 | 2012 | Ontario | AG-2-2IIIB |

[a]Isolate was collected by Dr. Sally Miller (Ohio State University) but curated in Dr. Darin Eastburn's laboratory (University of Illinois, Urbana-Champaign)

**Table 4.2.** List of isolates evaluated in the greenhouse assay for sensitivity to the SDHI and DMI fungicides.

| Isolate no | Isolate ID | $EC_{50}$ (µg/ml) | Sensitivity category[ab] |
|---|---|---|---|
| | **SDHI** | | |
| 1 | BVT-3 | 0.014 | SP |
| 2 | ER-15 | 0.081/0.184 | MSPS |
| 3 | K-IL-13C | 0.244 | LSPS |
| 4 | BVT-18 | 0.035 | SS |
| | **DMI** | | |
| 5 | EV-6 (AG-4) | 0.078 | SI |
| 6 | 42210b (AG-2-2) | 0.990 | MSI |
| 7 | DK-6 (AG-2-2) | 2.414 | LSI |
| 8 | BVT-28 (AG-4) | 0.239 | SPr |
| 9 | DK-15a (AG-2-2) | 1.514 | MSPr |
| 10 | DK-10 (AG-2-2) | 4.390 | LSPr |

[a]Since isolates could not be grouped into all four in vitro sensitivity categories, isolates in the low, medium, and high spectrum of sensitivity were selected for the in vivo study.

[b]Abbreviations: SP = sensitive to penflufen; MSPS = moderately sensitive to penflufen and sedaxane; LSPS: less sensitive to penflufen and sedaxane; SS = sensitive to sedaxane; SI = sensitive to ipconazole; MSI = moderately sensitive to ipconazole; LSI = less sensitive to ipconazole; SPr = sensitive to prothioconazole; MSPr = moderately sensitive to prothioconazole; LSPr = less sensitive to prothioconazole.

**Table 4.3.** Analysis of variance for $EC_{50}$ for the pre-2001 and post-2011 isolates of *Rhizoctonia solani* to penflufen, sedaxane, ipconazole and prothioconazole.

| | ***P* values** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Pre-2001 (n= 40)** | | | | | **Post-2011 (n=80)** | | | |
| **Source** | **df** | **Penflufen** | **Sedaxane** | **Ipconazole** | **Prothioconazole** | **df** | **Penflufen** | **Sedaxane** | **Ipconazole** | **Prothioconazole** |
| AG | 1 | < 0.0001 | < 0.0001 | < 0.0001 | 0.4716 | 4 | 0.016 | < 0.0001 | < 0.0001 | 0.0001 |
| Trial | 1 | 0.9651 | 0.6669 | 0.3401 | 0.4587 | 1 | 0.265 | 0.242 | 0.103 | 0.192 |
| Trial × AG | 1 | 0.176 | 0.613 | 0.6011 | 0.611 | 4 | 0.862 | 0.181 | 0.639 | 0.072 |
| Isolate (AG) | 38 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | 75 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |
| Residual | 118 | | | | | 235 | | | | |

**Table 4.4.** Mean and range of $EC_{50}$ values of the pre-2001 and post-2011 *Rhizoctonia solani* isolates to penflufen, sedaxane, ipconazole, and prothioconazole

| | No. of isolates | Penflufen[xy] | | | Sedaxane[xy] | | | Ipconazole[xy] | | | Prothioconazole[xy] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Range | SD[z] | Mean | Range | SD[z] | Mean | Range | SD[z] | Mean | Range | SD[z] |
| **Pre-2001** | | | | | | | | | | | | | |
| AG-2-2 | 27 | 0.056 a | 0.016-0.155 | 0.032 | 0.089 a | 0.029-0.215 | 0.043 | 1.338 b | 0.349-2.559 | 0.636 | 1.497 a | 0.242-3.554 | 0.904 |
| AG-4 | 13 | 0.017 b | 0.007-0.035 | 0.010 | 0.051 b | 0.026-0.119 | 0.025 | 1.894 a | 0.351-3.162 | 0.796 | 1.809 a | 0.200-3.665 | 1.026 |
| **Post-2011** | | | | | | | | | | | | | |
| AG-2-2 | 55 | 0.054 a | 0.028-0.244 | 0.030 | 0.103 b | 0.048-0.281 | 0.030 | 1.143 b | 0.148-3.434 | 0.554 | 2.328 a | 0.275-6.802 | 1.706 |
| AG-3 | 4 | 0.041 ab | 0.035-0.049 | 0.006 | 0.071 bc | 0.070-0.073 | 0.006 | 0.199 c | 0.132-0.365 | 0.101 | 0.435 b | 0.338-0.523 | 0.110 |
| AG-4 | 5 | 0.034 b | 0.014-0.043 | 0.013 | 0.064 c | 0.048-0.072 | 0.010 | 0.298 c | 0.078-0.463 | 0.146 | 0.493 b | 0.239-0.676 | 0.189 |
| AG-7 | 9 | 0.047 ab | 0.021-0.071 | 0.016 | 0.162 a | 0.095-0.263 | 0.067 | 0.495 c | 0.198-0.817 | 0.215 | 1.245 ab | 0.334-2.733 | 0.943 |
| AG-11 | 7 | 0.040 ab | 0.021-0.071 | 0.015 | 0.058 c | 0.022-0.129 | 0.015 | 1.797 a | 0.913-2.490 | 0.911 | 0.759 b | 0.283-1.791 | 0.538 |

[x]Units in µg/ml.

[y]Non-transformed values are reported.

[z]Standard deviation of the mean.

**Table 4.5**. Analysis of variance for the effects of fungicide seed treatment on plant stand, dried root weight, and disease severity indices of soybean treated with SDHI and DMI fungicides.

| Dependent variable | SDHI | | | DMI | | |
|---|---|---|---|---|---|---|
| | DF | MS | *P* > *F* | DF | MS | *P* > *F* |
| **Stand Count** | | | | | | |
| Isolate | 3 | 99900.0000 | 0.386 | 5 | 6445.6757 | 0.0011 |
| Fungicide | 2 | 898330.0000 | < 0.0001 | 2 | 12889.0000 | < 0.0001 |
| Isolate × fungicide | 6 | 137676.0000 | 0.037 | 10 | 297.7193 | 0.6188 |
| Block | 3 | 6451.4722 | 0.973 | 3 | 170.1190 | 0.8987 |
| Block x isolate | 9 | 88092.0000 | 0.156 | 15 | 881.3391 | 0.0051 |
| **Dry root weight** | | | | | | |
| Isolate | 3 | 0.0024 | 0.325 | 5 | 0.0227 | 0.0003 |
| Fungicide | 2 | 0.0328 | < 0.0001 | 2 | 0.0541 | 0.0005 |
| Isolate × fungicide | 6 | 0.0052 | 0.016 | 10 | 0.0034 | 0.8847 |
| Block | 3 | 0.0003 | 0.918 | 3 | 0.0023 | 0.4563 |
| Block × isolate | 9 | 0.0018 | 0.463 | 15 | 0.0024 | 0.9851 |
| **Disease severity index** | | | | | | |
| Isolate | 3 | 0.4146 | 0.1584 | 5 | 1.3517 | < 0.0001 |
| Fungicide | 2 | 32.7085 | < 0.0001 | 2 | 5.7827 | < 0.0001 |
| Isolate × fungicide | 6 | 0.8796 | 0.0639 | 10 | 0.8768 | < 0.0001 |
| Block | 3 | 0.3785 | 0.1841 | 3 | 0.0291 | 0.8462 |
| Block × isolate | 9 | 0.1889 | 0.9024 | 15 | 0.1073 | 0.8148 |

**Table 4.6.** Effects of fungicide seed treatments on disease severity index, stand count, and dried root weight of soybean seeds treated with SDHI and DMI fungicides.

| Fungicide Class | Isolate[y] | Treatment | DSI (0-1)[z] | Stand count[z] | Root weight (mg) [z] |
|---|---|---|---|---|---|
| **SDHI** | | | | | |
| | 1 | Penflufen | 0.28 b | 9 a | 0.195 a |
| | (SP) | Sedaxane | 0.27 b | 10 a | 0.182 a |
| | | No treatment | 0.63 a | 8 b | 0.117 b |
| | 2 | Penflufen | 0.32 b | 8 b | 0.135 b |
| | (MSPS) | Sedaxane | 0.28 b | 10 a | 0.197 a |
| | | No treatment | 0.75 a | 8 b | 0.116 b |
| | 3 | Penflufen | 0.29 b | 10 a | 0.192 a |
| | (LSPS) | Sedaxane | 0.35 b | 9 a | 0.165 a |
| | | No treatment | 0.59 a | 9 a | 0.163 a |
| | 4 | Penflufen | 0.34 b | 10 a | 0.168 a |
| | (SS) | Sedaxane | 0.30 b | 9 a | 0.203 a |
| | | No treatment | 0.54 a | 7 b | 0.109 b |
| **DMI** | 5 | Ipconazole | 0.73 a | 6 a | 0.324 a |
| | (SI) | Prothioconazole | 0.49 b | 7 a | 0.374 a |
| | | No treatment | 0.76 a | 1 b | 0.200 b |
| | 6 | Ipconazole | 0.48 b | 8 a | 0.410 a |
| | (MSI) | Prothioconazole | 0.42 b | 9 a | 0.390 a |
| | | No treatment | 0.68 a | 7 b | 0.346 a |
| | 7 | Ipconazole | 0.52 b | 9 a | 0.438 a |
| | (LSI) | Prothioconazole | 0.45 b | 10 a | 0.410 a |
| | | No treatment | 0.78 a | 8 b | 0.360 a |
| | 8 | Ipconazole | 0.42 b | 9 a | 0.432 a |
| | (SPr) | Prothioconazole | 0.44 b | 9 a | 0.401 a |
| | | No treatment | 0.50 a | 7 b | 0.372 a |
| | 9 | Ipconazole | 0.36 b | 9 a | 0.411 a |
| | (MSPr) | Prothioconazole | 0.56 a | 10 a | 0.407 a |
| | | No treatment | 0.67 a | 7 b | 0.349 a |
| | 10 | Ipconazole | 0.52 b | 10 a | 0.404 a |
| | (LSPr) | Prothioconazole | 0.53 b | 9 a | 0.391 a |
| | | No treatment | 0.79 a | 8 b | 0.355 a |

[y]Abbreviations: SP = sensitive to penflufen; MSPS = moderately sensitive to penflufen and sedaxane; LSPS: less sensitive to penflufen and sedaxane; SS = sensitive to sedaxane; SI =

**Table 4.6 (cont.)**

sensitive to ipconazole; MSI = moderately sensitive to ipconazole; LSI = less sensitive to ipconazole; SPr = sensitive to prothioconazole; MSPr = moderately sensitive to prothioconazole; LSPr = less sensitive to prothioconazole.

[z] Means with the same letter are not significantly different using Fisher's protected least significant difference (LSD, $\alpha = 0.05$)

**Table 4.7.** Percentages of *Rhizoctonia solani* isolates of the pre-2001 and post-2011 groups within each in vitro sensitivity category

| Fungicide | Category[a] | Percentage of isolates | |
|---|---|---|---|
| | | pre-2001 | post-2011 |
| Penflufen | Highly sensitive | 7.5 | - |
| | Sensitive | 87.5 | 98.75 |
| | Moderately sensitive | 5 | 1.25 |
| | Less sensitive | - | - |
| Sedaxane | Highly sensitive | - | - |
| | Sensitive | 80 | 61.25 |
| | Moderately sensitive | 20 | 38.75 |
| | Less sensitive | - | - |
| Ipconazole | Highly sensitive | - | - |
| | Sensitive | - | 1.25 |
| | Moderately sensitive | 22.5 | 51.25 |
| | Less sensitive | 77.5 | 47.5 |
| Prothioconazole | Highly sensitive | - | - |
| | Sensitive | - | - |
| | Moderately sensitive | 30 | 43.75 |
| | Less sensitive | 70 | 56.25 |

[a]Highly sensitive ($EC_{50}$ < 0.01 µg/ml); sensitive (0.01< $EC_{50}$ < 0.1 µg/ml); moderately sensitive (0.1<$EC_{50}$ < 1 µg/ml); and less sensitive ($EC_{50}$ ≥1 µg/ml).

**Figures**

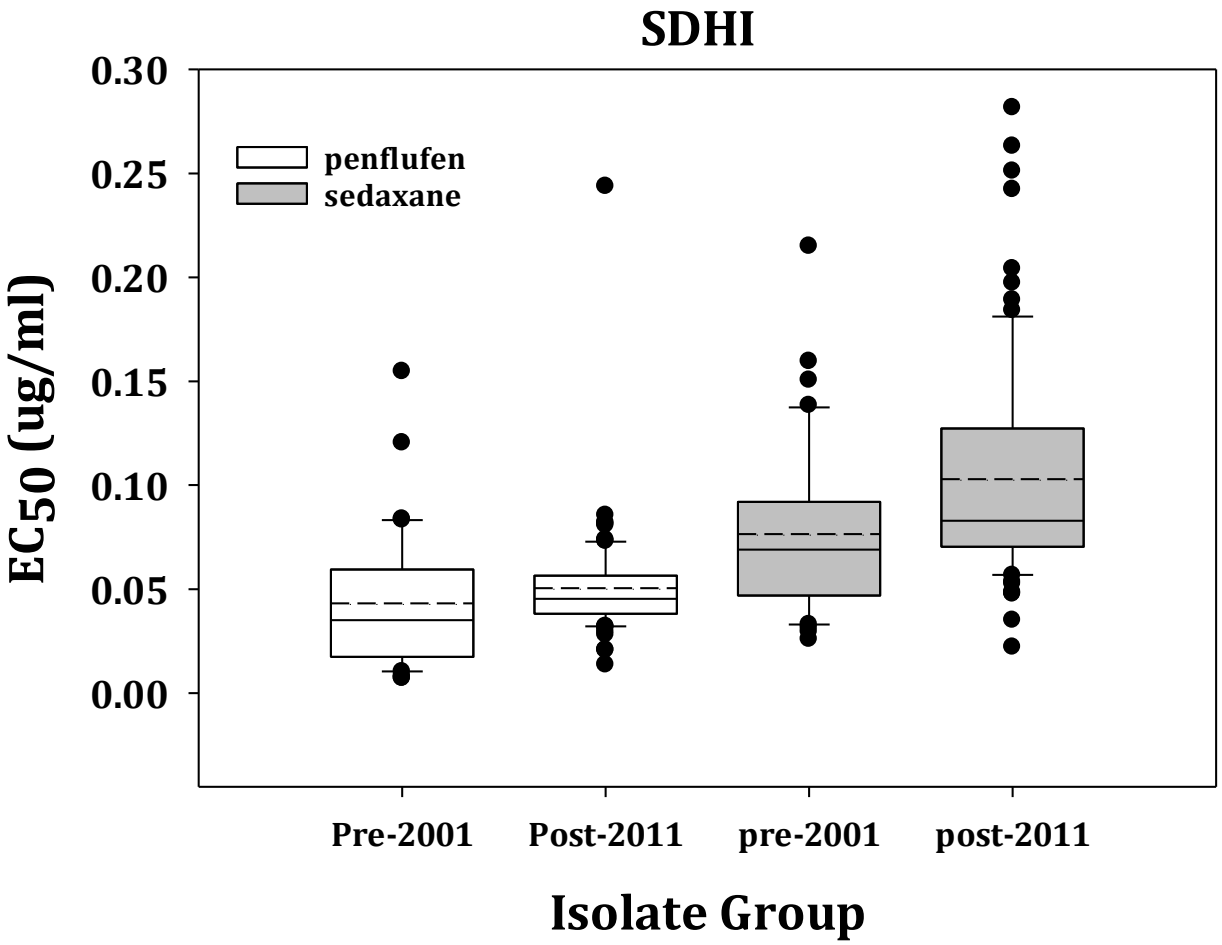


**Fig. 4.1**: Overall distribution of $EC_{50}$ values of the pre-2001 and post-2011 *Rhizoctonia solani* isolates for the SDHI fungicides. (Dashed lines represent mean $EC_{50}$ value for each fungicide. Solid lines represent median of distribution).

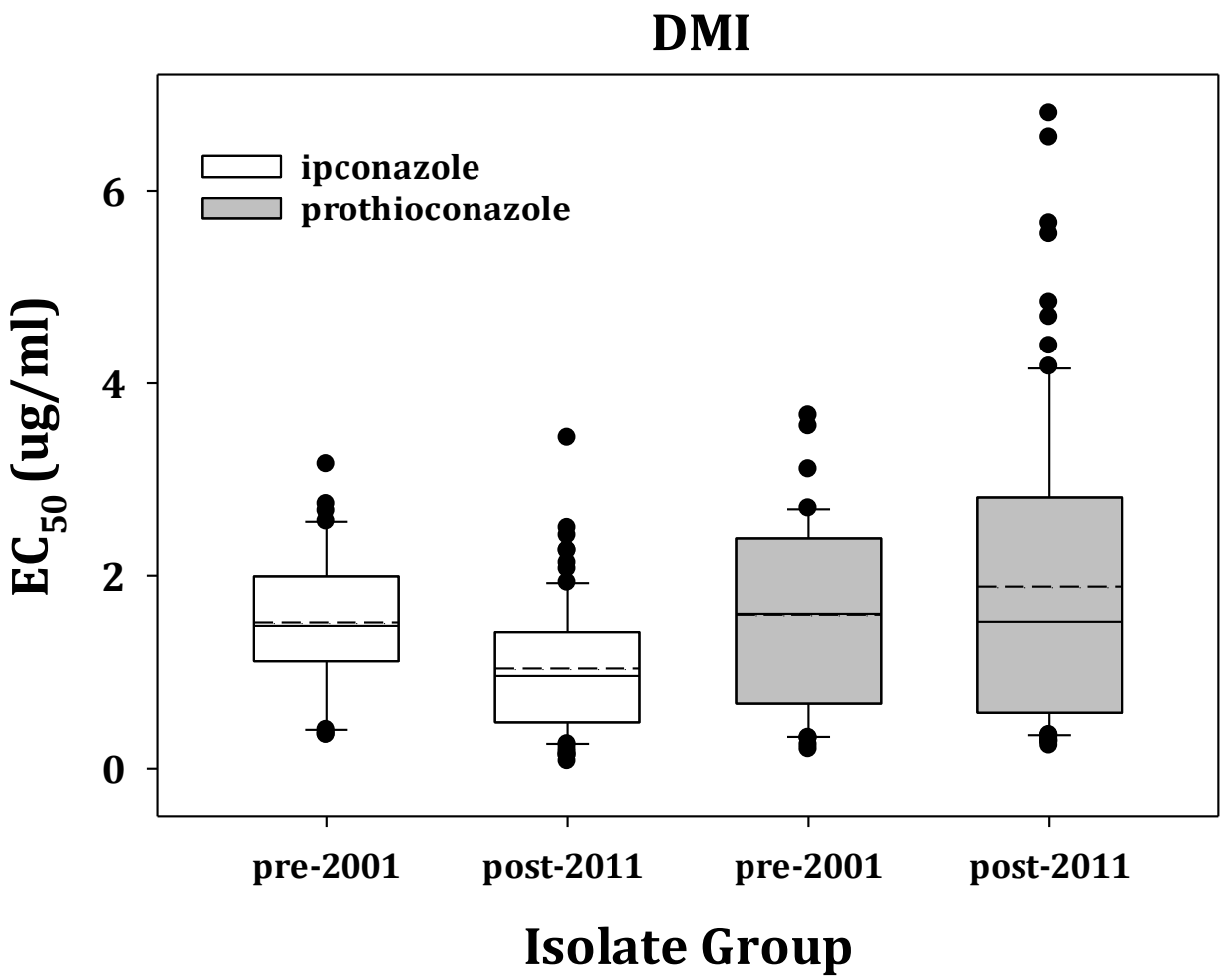


**Fig. 4.2**. Overall distribution of $EC_{50}$ values of the pre-2001 and post-2011 *Rhizoctonia solani* isolates for the DMI fungicides. (Dashed lines represent mean $EC_{50}$ value for each fungicide. Solid lines represent median of distribution)

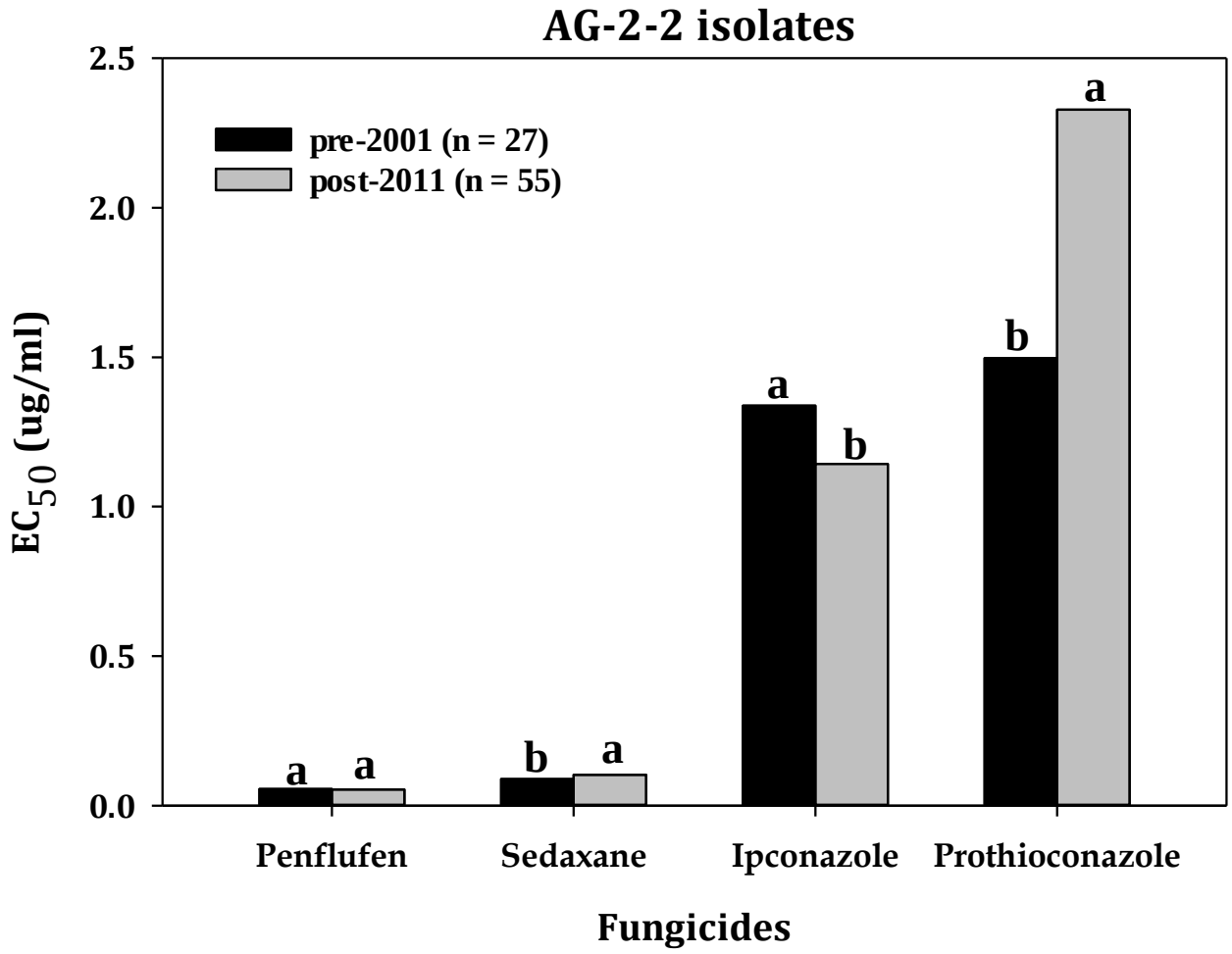


**Fig. 4.3**. Mean $EC_{50}$ values of pre-2001 and post-2011 isolates of *Rhizoctonia solani* AG-2-2. For each fungicide, bars with the same letter are not significantly different using Fisher's protected least significant difference (LSD, $\alpha = 0.05$)

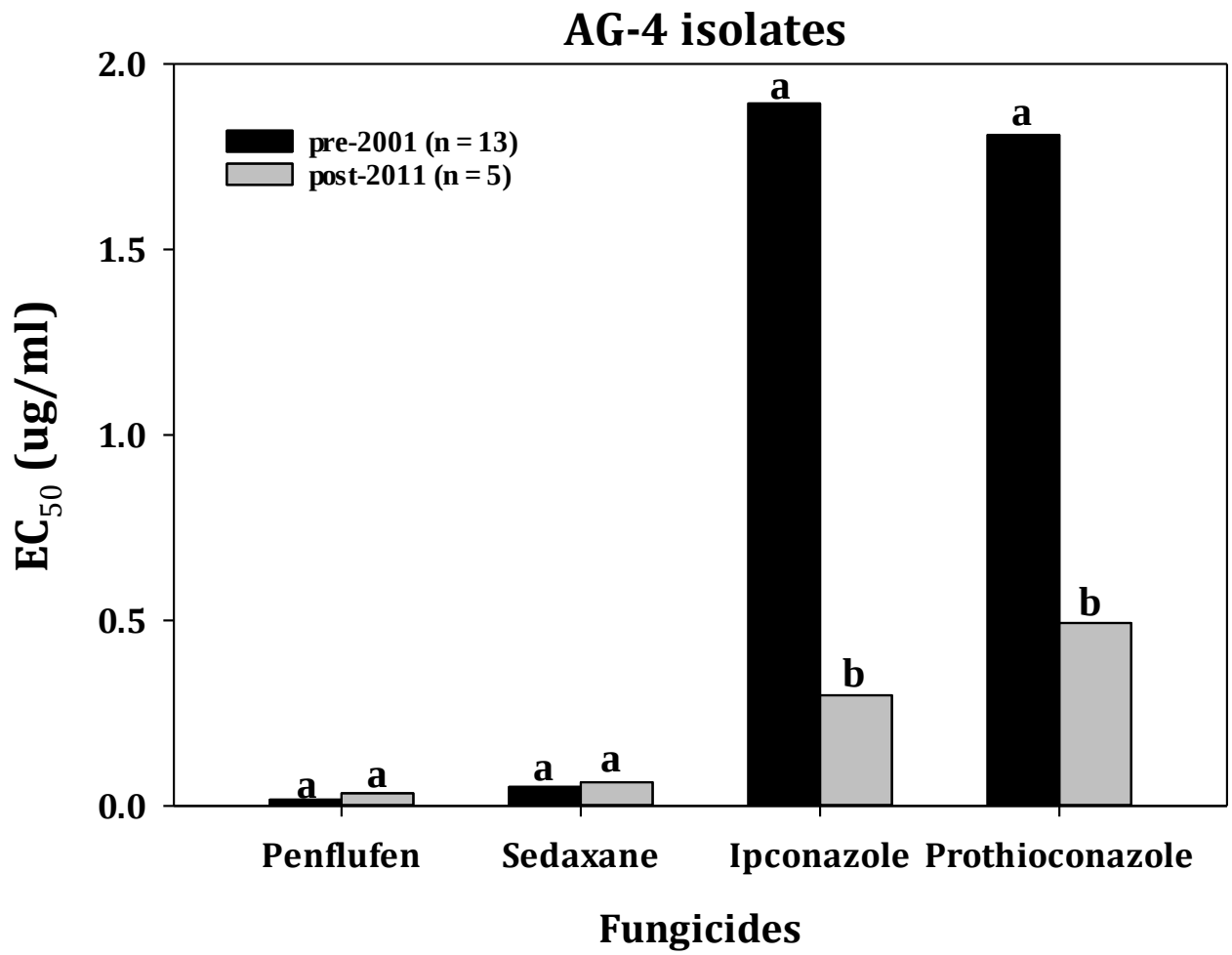


**Fig. 4.4**. Mean $EC_{50}$ values of pre-2001 and post-2011 isolates of *Rhizoctonia solani* AG-4. For each fungicide, bars with the same letter are not significantly different using Fisher's protected least significant difference (LSD, $\alpha = 0.05$)

# APPENDIX: DIFFERENTIAL REACTIONS OF SOYBEAN ISOLINES WITH COMBINATIONS OF APHID RESISTANCE GENES *RAG1, RAG2,* AND *RAG3* TO FOUR SOYBEAN APHID BIOTYPES[1]


## Abstract

With the discovery of the soybean aphid (*Aphis glycines* Matsumura) as a devastating insect pest of soybean (*Glycine max* (L.) Merr.) in the United States, host resistance was recognized as an important management option. However, the identification of soybean aphid isolates exhibiting strong virulence against aphid resistance genes (*Rag* genes) has highlighted the need for pyramiding genes to help ensure the durability of host resistance as a control strategy. In this study, soybean isolines with all possible combinations of the resistance and susceptibility alleles at *Rag1*, *Rag2*, and *Rag3* were evaluated for their effectiveness against the four characterized soybean aphid biotypes. All soybean isolines, including the susceptible check carrying none of the resistance alleles (S1/S2/S3), were infested with each biotype in no-choice greenhouse tests, and the aphid populations developed on each isoline were enumerated 14 days after infestation. All gene combinations, with the exception of *Rag3* alone, provided excellent protection against biotype 1. Isolines with *Rag2* alone or in combination with *Rag1* and *Rag3* had greater levels of resistance to biotype 2 than those with either *Rag1* alone, *Rag3* alone, or the *Rag1/3* pyramid. For biotype 3, the *Rag1/3* and *Rag1/2/3* pyramided lines significantly reduced aphid populations compared to all other gene combinations, while the *Rag1/2/3* pyramid provided the greatest protection against biotype 4. Overall, the *Rag1/2/3* pyramided line conferred the greatest protection against all four biotypes.



Keywords: *Aphis glycines*, biotypes, soybean


[1]This chapter has been accepted for publication by the Journal of Economic Entomology

## Introduction

The soybean aphid, *Aphis glycines* Matsumura, is a destructive insect pest of soybean (*Glycine max* (L.) Merr.). Although native to Asia, its first occurrence in the United States dates back to 2000 (Hartman et al. 2001) and its distribution has since expanded to almost all soybean growing-regions in the United States and Canada (Venette and Ragsdale 2004). The agronomic and economic impacts of the soybean aphid on soybean are significant. Feeding injury can result in stunting, leaf distortion, and reductions in the number of seed pods (Ragsdale et al. 2007; Sun et al. 1990). In addition, the photosynthetic potential of infected plants may be significantly impacted by the colonization of sooty molds on soybean leaves covered with honey dew excreted by feeding aphids (Gomez et al. 2006; Macedo et al. 2003). Furthermore, yield losses, which have been associated with premature pod abscission due to insect feeding during R1 through R4 growth stages (Fehr et al. 1971), can be as high as 50% in the United States (Ragsdale et al. 2006) or even greater in other parts of the world (He et al. 1991; Wang et al. 1994). The indirect impacts of soybean aphids are evident in their ability to efficiently vector plant viruses, including *Soybean mosaic virus* (Domier et al. 2003; Hartman et al. 2001; Hill et al. 2001).

Timely foliar insecticide applications, especially when the economic threshold of 250 aphids per plant has been reached and more than 80% of plants have become infested (Ragsdale et al. 2006; Ragsdale et al. 2007), can prevent yield losses (Hartman et al. 2011). However, given the increase in production costs associated with insecticide use (Ragsdale et al. 2007), the threat of insecticides to beneficial insects (Desneux et al. 2007; Theiling and Croft 1988) and the environment (van der Werf 1996), as well as the potential for insecticide resistance with repeated applications, host resistance continues to serve as the most important and environmentally-sound control tactic.

In North America, several sources of aphid resistance have been identified in accessions from the USDA Soybean Germplasm Collection (Hill et al. 2004; Mensah et al. 2005; Mian et al. 2008a). Resistance has been characterized, using choice and no-choice experiments, as antibiosis, which affects the insect's biology by interfering with its growth and reproductive ability; antixenosis, which affects the insect's behavior and is expressed as a non-preference for a specific host; or as tolerance, which confers the ability to withstand devastating insect populations (Smith 2005).

The genetic basis of resistance has been investigated, and a number of aphid resistance genes have been named (Hill et al. 2012). Resistance in the soybean cultivars Jackson and Dowling to an Illinois aphid isolate was found to be inherited as single dominant genes, and were named *Rag* and *Rag1*, respectively (Hill et al. 2006a; Hill et al. 2006b). Both genes were later mapped to the same chromosomal location [chromosome 7; linkage group (LG) M] (Li et al. 2007), suggesting they carry resistance at the same locus or at different closely linked loci (Hill et al., 2012). The identification and mapping of the second aphid resistance gene coincided with the discovery of a new soybean aphid biotype from Wooster, OH. Previous studies showed that the Illinois aphid isolate was unable to colonize plants with *Rag1* (Hill et al. 2004, 2006a), but the Wooster isolateswere found to densely colonize plants with *Rag1* (Kim et al. 2008); hence, the Illinois isolate was named biotype 1, while the Ohio isolate was designated biotype 2 (Hill et al. 2009).

Resistance to both biotype 1 and 2 was identified in Plant Introduction (PI) lines PI 243540 (Kang et al. 2008; Mian et al. 2008a; Mian et al. 2008b) and PI 200538 (Hill et al. 2009), and the underlying resistance gene from the two sources was mapped to the same location on chromosome 13 (LG F) and was named *Rag2*. Unfortunately, SF-55, an aphid isolate recovered

from Springfield Fen, Indiana, was found to colonize plants with *Rag2*, leading to the designation of biotype 3 (Hill et al. 2010). SF-55 is highly virulent on soybean genotypes with *Rag2* and can also colonize plants with *Rag1* and *Rag1*/*Rag2* in choice and no-choice experiments, indicating that a stronger antibiosis-type resistance is needed for the long-term management of this biotype (Hill et al. 2010). Unfortunately, no resistance gene with complete antibiosis or antixenosis-type resistance has been reported for this biotype. More recently, a soybean aphid from Lomira, WI was designated biotype 4 (Alt and Ryan-Mahmutagic 2013). This biotype was found to be highly virulent on plants with *Rag1*, *Rag2*, and the *Rag1/Rag2* pyramid (Alt and Ryan-Mahmutagic 2013). Similar to biotype 3, sources of resistance and *Rag* genes specific to biotype 4 have not been reported.

With the identification of different aphid biotypes, additional aphid resistance loci were sought, and a third aphid resistance gene, *Rag3*, was mapped in PI 567543C (Zhang et al. 2010), a soybean accession that was reported to express antixenosis-type resistance against aphid isolates found near East Lansing, Michigan (Mensah et al. 2005). Five additional soybean aphid resistance genes have been further characterized. Of these, *Rag3b* (Zhang et al. 2013), and *Rag5* (Jun et al. 2012; Mian et al. 2008a), are dominant, while the remaining three, *rag1b* (Bales et al. 2013; Mensah et al. 2008), *rag1c* (Zhang et al. 2010), and *rag4* (Zhang et al. 2009), are recessive. While there are no reports of the results from testing these additional genes with the four characterized aphid biotypes, they expand the range of resistance genes available to breeders for developing aphid-resistant soybean cultivars. Of the eight known aphid resistance genes, only *Rag1* and the *Rag1*/*Rag2* pyramid are currently deployed in commercial soybean cultivars marketed as having resistance to the soybean aphid (Caspers-Simmet 2008; McCarville et al. 2012). Given the virulence diversity in the population of soybean aphids in North America

(Cooper et al. 2015), the variability in aggressiveness among isolates of any one biotype (Pawlowski et al. 2015), and the ability of aphids to move large distances, durable resistance may be best achieved by pyramiding multiple *Rag* genes. As the arsenal of deployable aphid resistance genes with antibiosis and antixenosis-type resistance continues to expand, the options available to breeders are enormous, but the efficacy of gene combinations against the four known biotypes in North America has not been thoroughly investigated. In light of this knowledge gap, the objective of our research was to evaluate the differential reaction of soybean isolines carrying different combinations of the genes *Rag1*, *Rag2*, and *Rag3* to the four aphid biotypes identified in North America.

## Materials and Methods

**Aphid Culture Maintenance and Plant Materials**. Isolates of biotypes 1, 2, and 3 are clonal descendants of the original isolates from Illinois (Hill et al. 2004, 2006a, 2006b), Ohio (Kim et al. 2008), and Indiana (Hill et al. 2010), respectively. Biotype 4 was obtained from Michael Crossley at the University of Wisconsin (Madison, WI). Although a different isolate from the Lomira isolate reported by Alt and Ryan-Mahmutagic (2013), the biotype 4 isolate was collected from a site near where the Lomira isolate was identified, and our greenhouse assays confirmed similar virulence patterns as the Lomira isolate. Colonies of the four biotypes have been continuously maintained in an apterous state in isolated growth chambers after the original collections were made, which would be at least several hundred generations devoid of sexual reproduction. These biotypes have also been periodically cloned and tested to confirm virulence spectrums.

Separate pilot choice tests were initially set up to confirm the identity of each biotype and to subsequently monitor the virulence expression of each biotype on differential hosts on which

they had been maintained. Briefly, for biotypes 1 and 2, two plants each for soybean genotypes Williams 82 (no *Rag* gene), LD10- 5903a (*Rag1*), and LD08-12435a (*Rag2*) were sown in a triangular pattern into 15 cm plastic pots containing a soilless potting medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA, USA) and placed in a greenhouse maintained at 25°C. As the plants approached VC growth stage (Fehr et al. 1971), plants were thinned to one of each genotype per pot. A detached leaf from a previously infected plant containing multiple life stages was placed in the middle of the pot, and the levels of aphid colonization on each genotype were monitored after 14 d. A similar design was adopted for biotypes 3 and 4, but in this case, Williams 82 was replaced by LD12-12734a, a soybean breeding line with the *Rag1*/*Rag2* pyramid. Williams 82 was excluded from the virulence confirmation experiment for these biotypes since previous work had shown that *Rag2* was as susceptible as Williams 82 when infested with biotype 3 (Hill et al. 2010) and because biotype 4 is virulent on all *Rag* gene combinations (Alt and Ryan-Mahmutagic, 2013). Confirmation of virulence for the four biotypes was by visual observation of the number of aphids on each genotype.

Eight soybean breeding lines differing in *Rag* gene combinations (Table 1) were used in the main experiment. These lines were developed through four backcrosses using markers to select for the resistance genes during each generation of backcrossing. LD02-4485 served as the recurrent parent, while Dowling was the donor of *Rag1*, PI 200538 the donor of *Rag2*, and PI 567543C the donor of *Rag3*.

**Experimental Set-up and Statistical Analysis.** To determine the interaction between the eight soybean isolines and the four soybean aphid biotypes, no-choice tests were conducted as a factorial experiment arranged in a completely randomized design (CRD). Protocols for infestation were similar to those described by Hill et al. (2010). Briefly, two seeds of each isoline

were sown into 15 cm plastic pots filled with soilless potting medium (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA, USA) in a greenhouse maintained at a 14 h photoperiod with temperatures ranging between 22°C and 25°C. Plants in each pot were thinned to one as all plants approached VC stage. Using a damp script liner brush (Royal and Langnickel, Munster, IN), 10 aphid nymphs ($2^{nd}$ to $3^{rd}$ instar) were carefully placed on the adaxial side of one of the expanding unifoliate leaves at the VC stage. To prevent migration of aphids from infested plants, each pot was covered with a 100- by 300 mm plastic cylindrical cage having a 4-mm wall thickness and two 80- by 180 mm side windows of dimensions sealed with a silk fabric material with 0.1-mm apertures (Hill et al. 2010). Each infested plant represented an experimental unit, and each unit was replicated three times to give a total of 96 experimental units (8 isolines x 4 biotypes x 3 replications). Aphid colonization was evaluated 14 days post infestation by counting the number of aphids on each plant. The no-choice experiment was repeated in a second trial in the same greenhouse and under the same environmental conditions as the first trial.

The genotype of each isoline was confirmed by conducting a TaqMan assay. For this analysis, two representative seedling samples of each isoline that were randomly selected from the thinned plants prior to aphid infestation were transplanted into a soilless potting medium in the greenhouse maintained at 25°C. At the V2 to V3 growth stage, the uppermost fully expanded trifoliolate leaves were sampled for each isoline, and DNA was extracted using the CTAB method as described by Keim and Shoemaker (1988). SNP marker analysis was carried out as described by Kaczorowski et al. (2008) using a LightCycler 480 System (Roche Diagnostics, Indianapolis, IN). Williams 82, Dowling, PI 200538, and E10005 served as reference genotypes for no *Rag* gene, *Rag1*, *Rag2*, and *Rag3*, respectively. SNP markers used for genotyping include

22289 (*Rag1*) (Kim et al. 2010a), KS12 (*Rag2*) (Kim et al. 2010b), and MSUSNP16-10 (*Rag3*) (Bales et al. 2013; Zhang 2012)

Aphid count data for each trial were log transformed to ensure normal distribution and homogeneity of residuals before analysis in SAS (PROC GLM, SAS Institute 2001, Cary, NC). Normality of the data was confirmed after transformation by using the *p*-value obtained from the Shapiro-Wilk normality test and by visual observation of the Q-Q plots. A Brown-Forsythe test of homogeneity of variance was conducted to confirm homogeneity of variance for the residuals after transformation and to determine if trials could be pooled before analysis. Contrast statements were used to determine significant differences among the eight soybean isolines for each of the four aphid biotypes. Although non-transformed data are presented, mean separations reported are from the analysis of the transformed values.

To determine the similarity in the reaction of the four soybean aphid biotypes to the different gene combinations, aphid count data were subjected to hierarchical cluster analysis in R (R Core Team, 2015). For analysis, distance matrix was computed using the Euclidean metric in the dist function (*stats* package), and the Ward's minimum variance method (ward.D2) was selected for agglomerative clustering in the hclust function (*stats* package).

**Results and Discussion**

The phenotypes of the soybean genotypes tested in the biotype confirmation pilot study were consistent with expectations (Table 2). Homogeneity of variance test for trial revealed a common variance for the residuals ($F = 2.01$; df = 1, 189; $P = 0.16$); therefore, the two trials were pooled for analyses. From the analysis of variance for the mean number of aphids, the main effects of trial, isoline, and biotypes were significant (Table 3). Significant interactions between isoline and biotype, and between trial and biotype were also detected (Table 3). All other

interaction effects were not significant. The observed significant interaction between trial and biotype resulted from the mean number of aphids observed being greater in trial 2 than trial 1, except for biotype 3 when a significantly greater numbers were observed in trial 1 than trial 2 (Table 4). The significant interaction between the soybean isolines and the aphid biotypes indicates a differential colonization of the isolines by the four aphid biotypes, thus confirming that the four aphid isolates used in this experiment were different biotypes. Multiple degree of freedom contrasts for the interactive effect of isoline by biotype showed that the differences in colonization among isolines for each biotype were highly significant ($P < 0.0001$); therefore, the differential reaction of the four biotypes to the eight isolines was reported for each biotype separately. Figures 1 to 4 provide a summary of the mean number of aphids recorded for each isoline across the two trials.

**Biotype 1**. Our results (Fig. 1) agree with those from previous studies that evaluated the differential response of soybean genotypes with *Rag1*, *Rag2*, and the *Rag1/2* to infestation by this biotype. Biotype 1 has been reported to be avirulent against *Rag1*, *Rag2*, or the *Rag1/2* gene combination (Hill et al. 2004, 2006a, 2006b, 2012). When compared to S1/S2/S3, *Rag1* produced significantly lower aphid numbers, which was not significantly different from that produced on *Rag2*. The aphid numbers recorded on *Rag3* alone were not significantly different from that on S1/S2/S3, suggesting that *Rag3* is ineffective against this biotype; however, when present in combination with *Rag1*, the aphid population was significantly reduced compared to *Rag1* alone. Contrary to what was observed when combined with *Rag1*, *Rag3* did not significantly improve the resistance conferred by *Rag2*. The lowest aphid number was recorded on the *Rag1/3* pyramid; however, the number wasn't significantly different from the aphid populations recorded on *Rag1/2*, *Rag2/3*, and *Rag1/2/3* stacks. These results showed that in all

the gene combinations evaluated, the presence of *Rag1* or *Rag2* ensured protection against biotype 1.

**Biotype 2**. Aphid populations were highest on S1/S2/S3, but the value was not significantly different from those obtained on *Rag1*, *Rag3*, and the *Rag1/3* pyramids (Fig. 2). The colonization of *Rag1* by this biotype agrees with previous findings (Kim et al. 2008); however, the differential colonization of this biotype on *Rag3* in no-choice tests was previously unknown. Our results showed the ineffectiveness of *Rag3* against this biotype when deployed only with *Rag1*. *Rag2* has been previously reported to provide strong antibiosis-type protection against biotype 2 (Kim et al. 2008), and this was confirmed in our test. Only the *Rag2* isoline or those with stacks that include this gene had less colonization than the susceptible line. The *Rag1/2/3* pyramid produced the lowest number of aphids for this biotype, but it was not significantly different than *Rag2* alone.

**Biotype 3**. Biotype 3 has been reported to overcome the resistance conferred by *Rag2* but is only able to colonize *Rag1* minimally (Alt and Ryan-Mahmutagic 2013; Hill et al. 2010; Pawlowski et al. 2015), and our results are in agreement with those findings (Fig. 3). The number of aphids observed on S1/S2/S3, although numerically higher, was not significantly different from those obtained on *Rag2* and *Rag3*. Aphid colonization on *Rag1* was significantly reduced when compared to the line with no *Rag* gene. We also observed that aphid colonization on *Rag1* alone or on the *Rag1/2* gene combination was numerically lower but not significantly different from the populations observed on *Rag2* and *Rag3*. The *Rag1/3* and *Rag1/2/3* pyramids provided the greatest protection against this biotype based on the significantly lower number of aphids observed.

**Biotype 4**. Compared to other biotypes, resistance genes showed the least effectiveness in controlling biotype 4. Aphid numbers produced on S1/S2/S3 were not significantly different from those observed on *Rag1, Rag2, Rag3, Rag1/2,* and *Rag1/3* (Fig. 4). Although the number of aphids produced on the *Rag1/2/3* pyramid was the lowest numerically, the value was not significantly different from those obtained from all resistance genes containing isolines except the one carrying *Rag1*. The three-fold decrease in aphid population observed on the triple pyramided line when compared to S1/S2/S3 suggests that the combination of all the three dominant genes provides the best protection against this biotype.

While the frequencies and distribution of soybean aphid biotypes across North America is unknown at present, the possibility of finding more than one aphid biotype within a geographical location cannot be completely ruled out. For instance, in eastern South Dakota, results from field evaluations of soybean genotypes with *Rag1* or *Rag2* under natural aphid infestations suggested the presence of at least two different biotypes (Bhusal et al. 2013). Different soybean aphid biotypes have also been reported in Michigan (Mensah et al. 2007). A 2-year study evaluating the geographic distribution of soybean aphid biotypes in the United States and Canada found considerable variability across states and years (Cooper et al. 2015). These observations across different soybean-growing states in North America highlight the importance of stacking aphid resistance genes to ensure the durability of host resistance as a management option for the soybean aphid control. Several field studies have evaluated the durability of lines carrying *Rag1, Rag2* or the *Rag1/2* pyramid to naturally occurring field isolates of the soybean aphid (McCarville and O'Neal 2012; McCarville et al. 2014; Wiarda et al. 2012). While the exact biotypic profiles of the aphid populations were unknown, there was consistency in the observation that a combination of *Rag1* and *Rag2* improved protection over either gene being

deployed alone. This suggests the presence of multiple biotypes, especially biotypes 1, 2, and 3. Our results indicate that the *Rag1/2* pyramid provides excellent protection against biotype 1 and biotype 2. Even though the colonization of biotype 3 on *Rag1/2* was statistically comparable to that of *Rag2*, biotype 3 was only able to infest *Rag1/2* minimally as it did *Rag1*. Hill et al. (2010) found a similarity in the response of soybean genotypes with *Rag1* and *Rag1/2* to colonization by biotype 3; however, the aphid populations on these genotypes were significantly lower than that observed on *Rag2*. While we are certain about the purity of the biotype 3 isolate used in this study, the comparable reaction of *Rag2* and *Rag1/2* to this biotype may be due to the significantly lower number of aphids produced by this biotype in the second trial (Table 4). It also is possible that the *Rag2* plants were environmentally stressed, which would have impeded the population growth of biotype 3.

Our results also provide evidence for the effectiveness of the *Rag1/3* gene combination against biotype 1 and biotype 3 but not against biotype 2 or biotype 4. Interestingly, the *Rag1/2/3* pyramided line provided the broadest range of protection against all four soybean aphid biotypes. For example, for biotypes 1 and 2, aphid populations were kept below the economic threshold level of 273 ± 38 aphids per plant (Ragsdale et al. 2007) throughout the 14-day period the aphids were allowed to feed; however, higher aphid numbers were recorded for biotypes 3 ( approximately 329 aphids) and 4 (approximately 677 aphids), albeit below the economic injury level of 674 ± 95 aphids per plant (Ragsdale et al. 2007). These findings suggests that in locations with very high frequencies of biotype 3 and biotype 4, yields of soybean lines carrying the *Rag1/2/3* pyramid could be threatened with rising populations of biotypes 3 and 4, which indicate the need for the identification of aphid resistance genes with stronger resistance against biotype 3 and biotype 4.

Since the goal of this study was to evaluate the effects of different aphid resistant gene combinations to the four known aphid biotypes, it was set up as a no-choice (factorial) experiment to allow us to take a closer evaluation at the interaction between these two factors (soybean isolines and soybean aphid biotypes). Generally, choice and no-choice tests are usually designed to identify antixenosis and antibiosis, respectively, even though a clear distinction between both resistance categories may not always be possible (Smith 2005). Antibiosis-type resistance has been identified as the primary resistance modality for *Rag1* (Hill et al. 2004; Li et al. 2004) and *Rag2* (Hill et al. 2009; Mian et al. 2008), although a few soybean genotypes with *Rag1* have been found to also express antixenosis-type resistance (Diaz-Montano et al. 2006; Hesler et al. 2007; Hesler and Dashiell 2011). *Rag3* was identified in PI 567543C, a soybean genotype that expresses predominantly antixenosis-type resistance based on the results obtained in no-choice test (Mensah et al. 2005). Therefore, the setup of this study might have prevented the *Rag3* expression of antixenosis when used alone. Even though *Rag3* did not appear to be effective against all four biotypes when used alone, in combination with the *Rag* genes effective against each respective biotype, it provided a stronger antibiosis-type resistance. For example, colonization of biotype 1 on *Rag1/3*, *Rag2/3*, and *Rag1/2/3* were lower than on *Rag1* or *Rag2* alone. Similarly, the combination of *Rag1* with *Rag3* provided a stronger protection against biotype 3 than just *Rag1* alone.

The observed ineffectiveness of *Rag3* alone against the four biotypes is not surprising since the resistance of PI 567543C to biotype 2 and two unidentified aphid isolates from Michigan was only confirmed in choice tests (Mensah et al. 2005; Mian et al. 2008; Zhang et al. 2010). In no-choice experiments conducted by Mensah et al. (2005), PI 567543C also did not provide the same level of protection as Jackson, a soybean genotype that is resistant to biotype 1.

Since we did not conduct a choice test, an evaluation of the antixenosis-type resistance of *Rag3* alone and in combination with other *Rag* genes is not possible. Our observations with *Rag3* highlights the importance of conducting preliminary tests to determine the biotype profile of any soybean isolate used in identifying resistance sources or mapping aphid resistance genes.

Results from hierarchical clustering (Fig. 5) provided information about the relationship among the four aphid biotypes in their reaction to the different combinations of *Rag* genes evaluated. Cluster analysis produced two clades; clade 1 consisted of biotype 1 and biotype 2, while clade 2 comprised biotype 3 and biotype 4. These results imply that biotype 1 and 2 exhibited similarity in their virulence to some or all of the eight soybean isolines carrying different combinations of *Rag* genes. Previous studies have indicated the inability of both biotypes 1 and 2 to colonize *Rag2* (Hill et al. 2010), and from our study, only gene combinations with *Rag2* proved effective against both biotypes. The similarity in avirulence against *Rag2* might explain the grouping of both biotypes within the same clade. The virulence expression of biotype 3 was comparable to that of biotype 4. Moreover, biotype 3 and biotype 4 both exhibited variability in their virulence patterns, which might explain the grouping of both biotypes within the same clade. Biotype 3 and biotype 4 are able to colonize *Rag1*, *Rag2*, and the *Rag1/2* pyramid (Alt and Ryan-Mahmutagic 2013; Hill et al. 2010), and it is unclear how they are different from each other. Compared to biotype 3, we observed higher populations of biotype 4 on all isolines, suggesting that the true differences between these two biotypes may be more related to their aggressiveness.

In conclusion, our results indicate that a three-gene pyramid has the potential for improved soybean aphid management and can thereby reduce the need for insecticide applications. Chandrasena et al. (2015) recently reported the effectiveness of *rag*3+*rag*1c gene

combination against field isolates from Michigan which, based on the phenotypes of differential hosts, were assumed to comprise both biotypes 3 and 4. Future work should be aimed at evaluating, under field conditions, the differential response of the *Rag1/2/3* pyramid to naturally infesting soybean aphid populations across field locations in North America, as well as the effect of *Rag1/2/3* pyramid on yield and other agronomic traits. Finally, a molecular explanation for the stronger antibiosis-type resistance provided by *Rag3* against a specific biotype when used in combination with *Rag* genes effective against that biotype would improve our understanding of the interaction between *Rag* genes and their potential for the management of different soybean aphid biotypes.

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## Tables

**Table A.1.** Soybean genotypes evaluated in no-choice experiment and their corresponding Rag gene combination

| Soybean isoline | Resistance gene |
|---|---|
| LD14-8001 | *Rag1/Rag2* (*Rag1/2*) |
| LD14-8002 | *Rag2* |
| LD14-8003 | *Rag1/Rag2/Rag3* (*Rag1/2/3*) |
| LD14-8004 | *Rag1* |
| LD14-8005 | *Rag1/Rag3* (*Rag1/3*) |
| LD14-8006 | *Rag3* |
| LD14-8007 | S1/S2/S3 |
| LD14-8008 | *Rag2/Rag3* (*Rag2/3*) |

**Table A.2**. Phenotypic expression of differential soybean genotypes after infestation with the four soybean aphid biotypes in the pilot study

| **Soybean differentials**[a] | **Biotypes**[b] | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Williams 82 (no *Rag* ) | + | + | | |
| LD10- 5903a (*Rag1*) | - | + | - | + |
| LD08-12435a (*Rag2*) | - | - | + | + |
| LD12-12734a (*Rag1/Rag2*) | | | - | + |

[a]Williams 82 was obtained from the USDA Soybean Germplasm Collection, Urbana, IL; breeding lines are from B. W. Diers' breeding program.

[b]+ and – indicate a virulent and avirulent reaction, respectively

**Table A.3.** Analysis of variance for the main and interactive effects of isoline and biotype on the number of aphids 14 days after infestation.

| Treatment effect | df | F value | *P* |
|---|---|---|---|
| Isoline | 7 | 31.72 | < 0.0001 |
| Biotype | 3 | 58.99 | < 0.0001 |
| Trial | 1 | 10.69 | 0.0014 |
| Isoline × biotype | 21 | 6.03 | < 0.0001 |
| Trial × isoline | 7 | 0.7 | 0.6744 |
| Trial × biotype | 3 | 6.94 | 0.0002 |
| Trial × isoline × biotype | 21 | 0.41 | 0.9895 |

**Table A.4.** Mean aphid counts by the four biotypes in the two trials

| | Trial 1 | Trial 2 | P value of mean difference |
|---|---|---|---|
| Biotype 1 | 343 | 568 | 0.0059 |
| Biotype 2 | 628 | 1089 | 0.0046 |
| Biotype 3 | 1063 | 785 | 0.0225 |
| Biotype 4 | 1023 | 1553 | 0.0021 |

## Figures

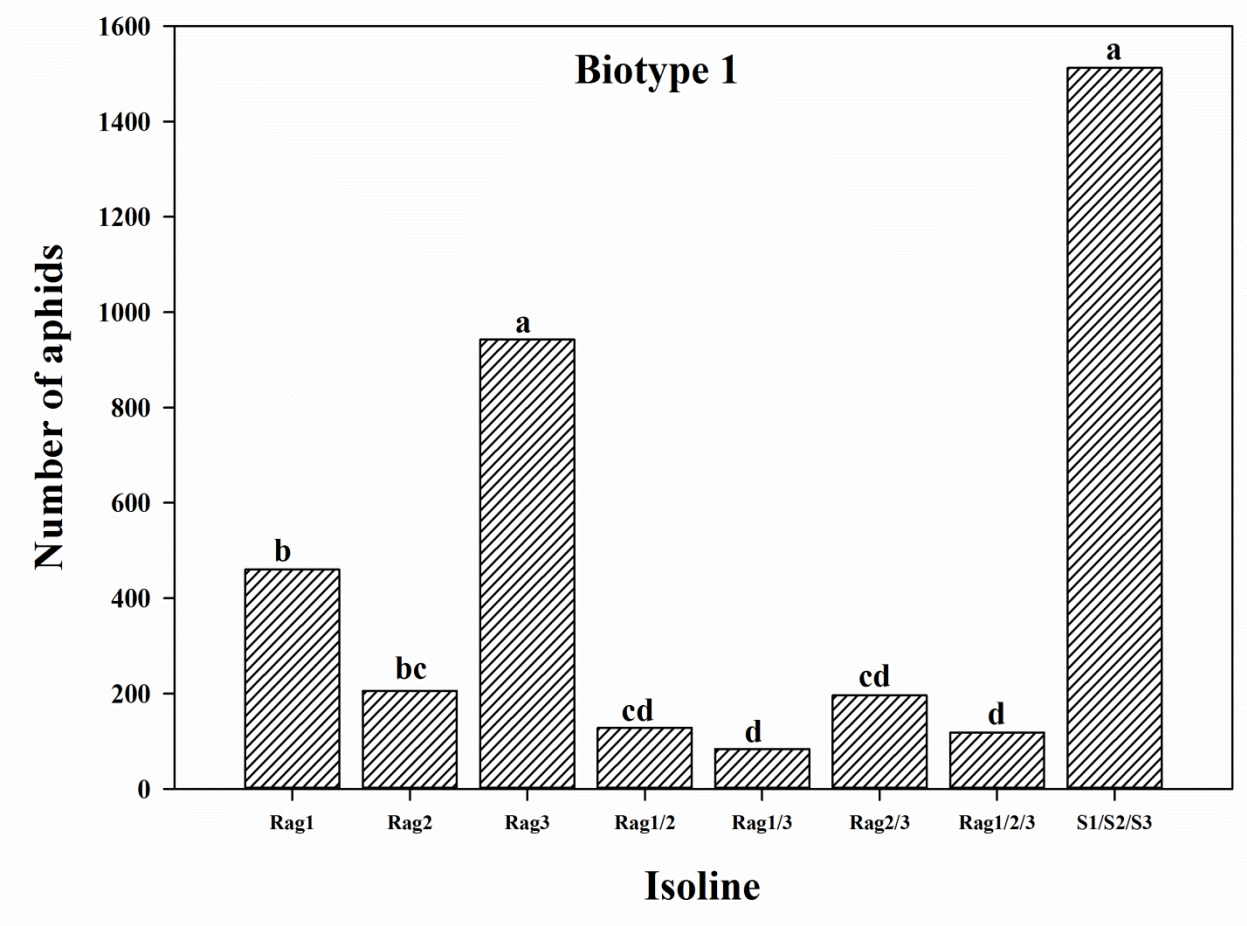


**Fig. A.1.** Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 1 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

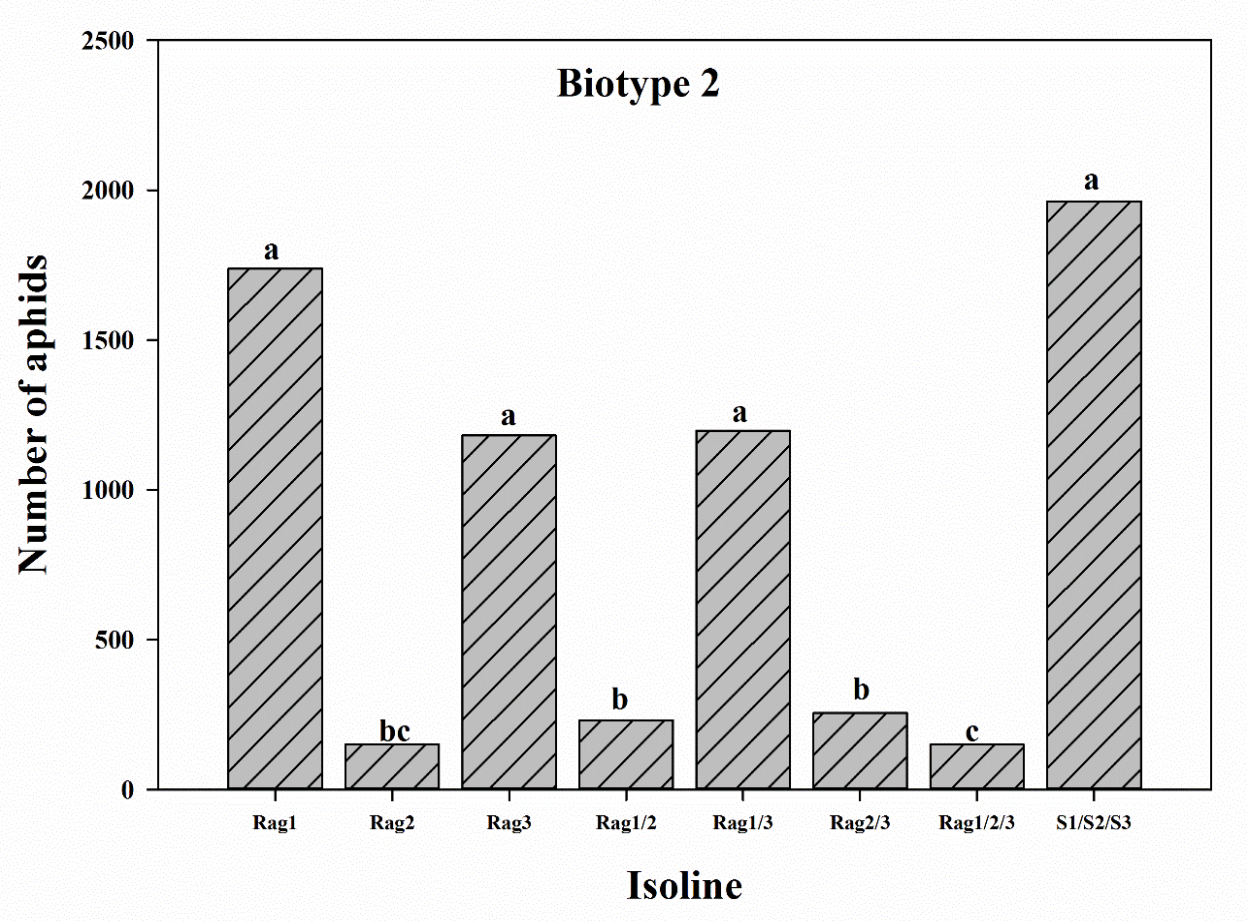


**Fig. A.2.** Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 2 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

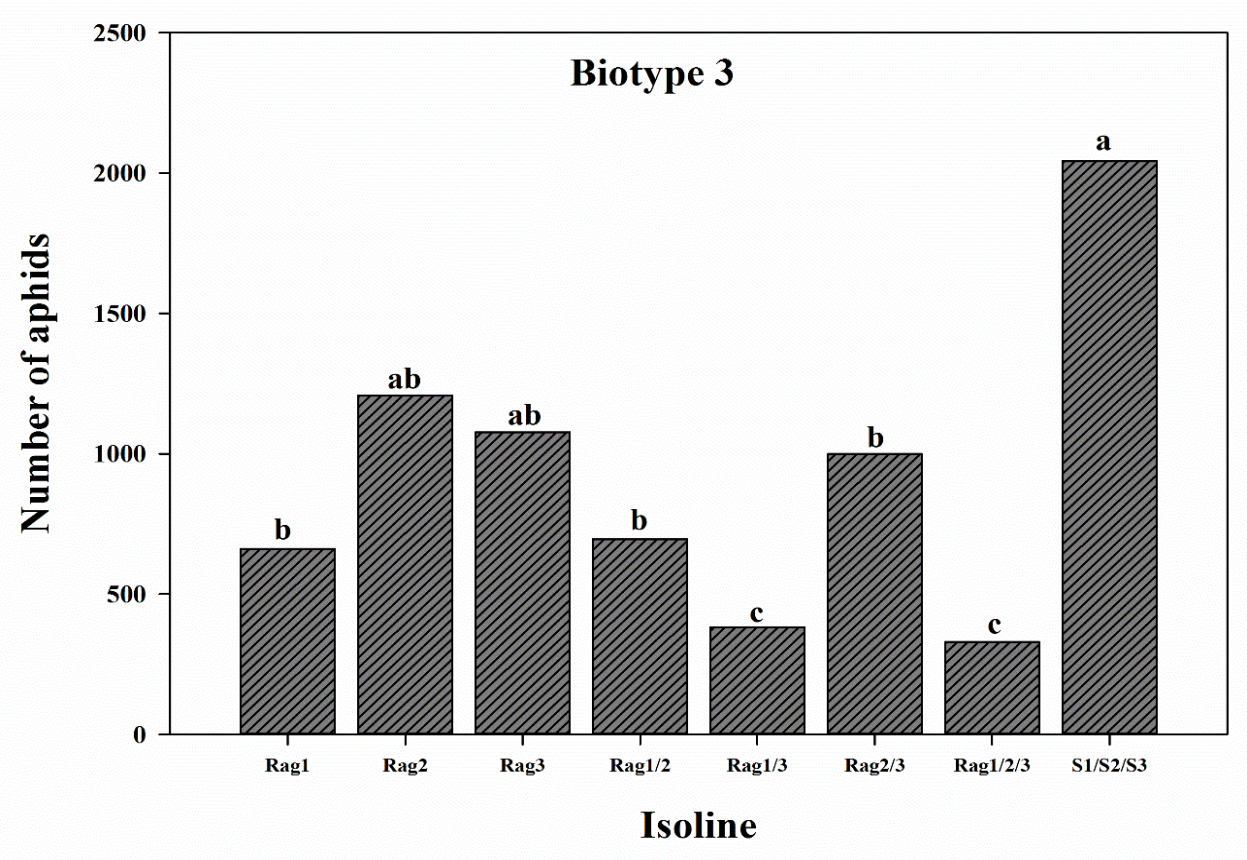


**Fig. A.3.** Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 3 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

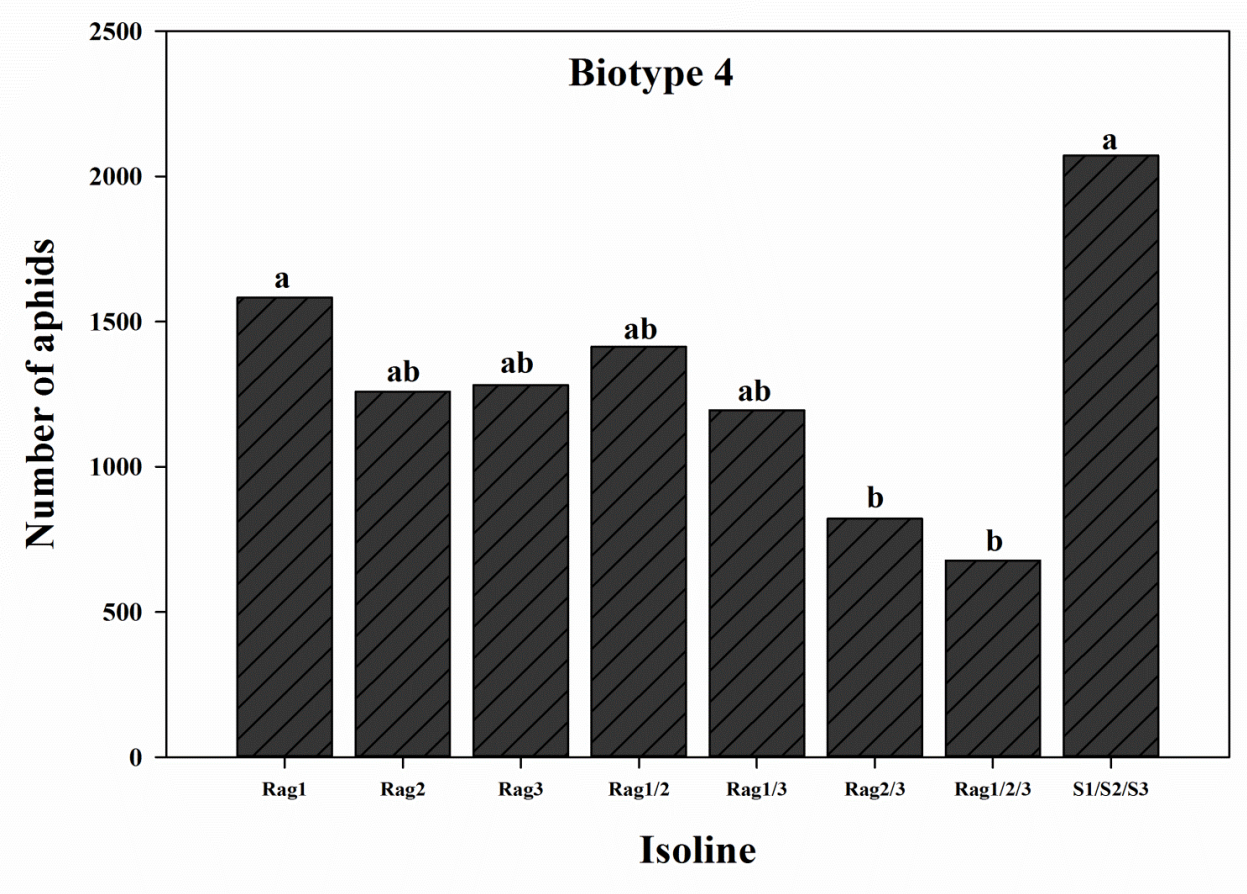


**Fig. A.4.** Differential colonization of the eight soybean isolines with combinations of *Rag* genes by soybean aphid biotype 4 across trials 1 and 2. Means with the same letter are not significantly different ($\alpha = 0.05$). Mean separations are from $\log_{10}$ transformed data.

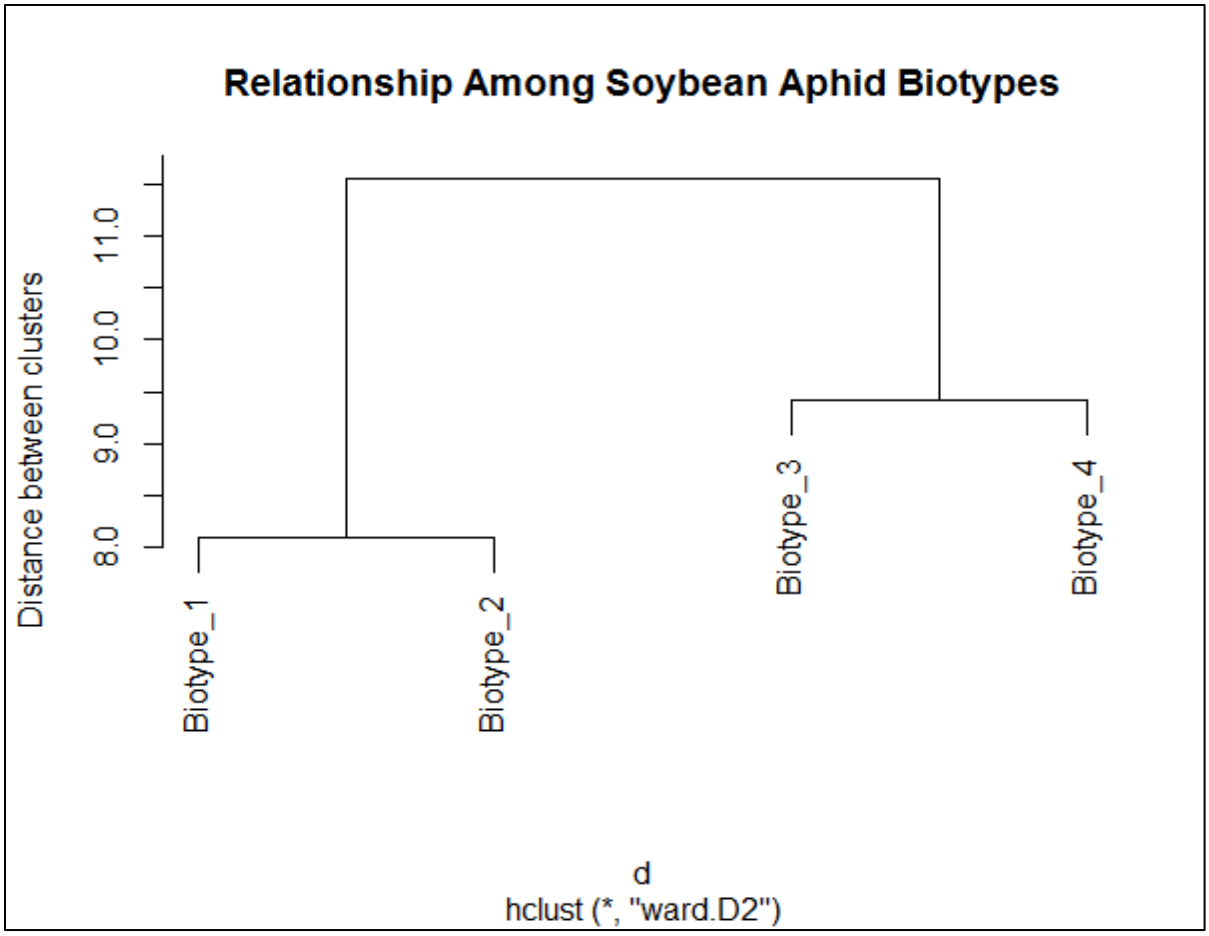


**Fig. A.5.** Cluster dendogram showing the relationship of the reactions of the soybean aphid biotypes to the different *Rag* gene combinations. Cluster analysis was conducted using the dist and hclust functions from the *stats* package in R.